

**University of Alberta**

**EVALUATION OF MYRISTIC ACID ANALOGS AND 5'-O-ESTER PRODRUGS  
OF THYMIDINE (dThd), 3'-AZIDO-3'-DEOXYTHYMIDINE (AZT) AND 3'-  
FLUORO-3'-DEOXYTHYMIDINE (FLT)**

by

**Keykavous Parang**



**A thesis Submitted to The Faculty of Graduate Studies and Research**

**in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy**

**in**

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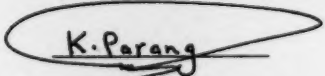
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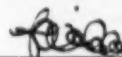
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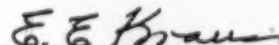
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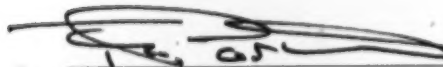
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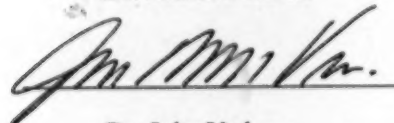
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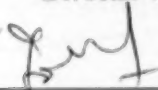
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**Dedicated to:**

**My father who taught me how to fight for my goals and my mother who taught me  
how to love**

## ABSTRACT

A series of prodrugs of zidovudine (AZT) and alovudine (FLT) has been synthesized in an effort to enhance their anti-HIV-1 activity, duration of action, lipophilicity, uptake by HIV-1 infected cells, delivery to the central nervous system and liver, and plasma half-life. Some of the other goals of this approach were to prevent opportunistic fungal infections, to improve drug transport, to reduce toxicity, and to overcome drug resistance.

The syntheses and pharmacological evaluation of myristic acid analogs and 5'-*O*-myristoyl analogue derivatives of nucleosides (AZT, FLT, dThd) have been investigated.

The myristic acid analogs were tested *in vitro* for activity against yeasts (*Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*), filamentous fungi (*Aspergillus niger*) and viruses (human immunodeficiency virus, hepatitis B virus, and duck hepatitis B virus).

Anti-HIV-1 activities of 5'-*O*-myristoyl analogs of thymidine (81a-h), zidovudine (82a-87) and alovudine (91a-93) analogs were determined against HIV-infected CD4<sup>+</sup> lymphocytes. All the prodrugs were more lipophilic than the parent compounds dThd (8), AZT (6), and FLT (7).

The structures of the 5'-*O*-myristoylthymidine analogs (81a-h) were optimized using molecular mechanics (MM<sup>+</sup> force field), and semi-empirical quantum mechanics (PM3), calculations. A quantitative structure-activity relationship (QSAR) regression equation was developed, based on the optimized structures and anti-HIV data using the *SciQSAR* computational program, which showed that Log P was a determinant of anti-HIV activity.

All of the AZT and FLT prodrugs (82a-87, 91a-93) exhibited potent activity against HIV. 3'-Azido-5'-*O*-(4-oxatetradecanoyl)-2',3'-deoxythymidine (82c) (EC<sub>50</sub> = 1.4 nM)

showed greater activity than AZT (**6**) ( $EC_{50} = 10.0$  nM) against HIV-infected CD4<sup>+</sup> lymphocytes in the CEM-SS cell line. The antiviral efficacy of some analogs were evaluated using a hepatitis virus B (HBV) DNA-transfected cell line 2.2.15 *in vitro*.

The stability of 5'-O-esters of dThd, AZT and FLT were determined following their *in vitro* incubation in the presence of porcine liver esterase, rat plasma and rat brain homogenate at 37 °C. Subsequent quantitative HPLC analyses indicated that these agents had a wide range of stability. For example, these myristoyl analog esters were cleaved by porcine liver esterase with a  $t_{1/2}$  in the range of 1.9 to 80.4 min.

The pharmacokinetics of 3'-azido-2',3'-dideoxythymidine (**6**,  $\beta$   $t_{1/2} = 112.5$  min; AUC =  $29.12 \pm 2.92$   $\mu\text{mol.g}^{-1}.\text{min}$ ; CL =  $10.47 \pm 1.05$   $\text{mL min}^{-1}.\text{kg}^{-1}$ ) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (**85b**,  $\beta$   $t_{1/2} = 428.5$  min; AUC =  $17.34 \pm 4.69$   $\mu\text{mol.g}^{-1}.\text{min}$ ; CL =  $17.58 \pm 4.76$   $\text{mL min}^{-1}.\text{kg}^{-1}$ ) were determined in male uninfected Balb/c mice (25-30 g). The *in vivo* results acquired indicate that the ester (**85b**) was rapidly converted to AZT *in vivo*. The biodistribution studies using selected compounds (**6**, **68c**, **85b**) demonstrated that **85b** provided AZT brain and liver concentrations greater than those measured following an equimolar doses of AZT. However, **85b** provided lower plasma levels of AZT, than observed after AZT administration. Mean brain/blood concentration ratios for AZT after prodrug ester administration ( $0.16 \pm 0.03$ ) were greater than those obtained after AZT injection ( $0.06 \pm 0.001$ ).

It is concluded that these analogs are potential prodrugs for the treatment of AIDS. This pharmaceutical approach would be beneficial for the efficient treatment of CNS and liver infections by human immunodeficiency virus (HIV) and/or hepatitis B virus (HBV).

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## LIST OF ABBREVIATIONS

|                 |                                                                                                             |
|-----------------|-------------------------------------------------------------------------------------------------------------|
| ADC             | AIDS dementia complex                                                                                       |
| AIDS            | Acquired immunodeficiency syndrome                                                                          |
| AM1             | Austin model 1. A semi-empirical method for molecular calculations                                          |
| AMT             | 3'-Amino-3'-deoxythymidine                                                                                  |
| <i>Anal</i>     | Analysis                                                                                                    |
| <i>A. niger</i> | <i>Aspergillus niger</i>                                                                                    |
| ARC             | AIDS-related complex                                                                                        |
| Arfs            | ADP-ribosylation factors                                                                                    |
| AUC             | Area under the curve                                                                                        |
| AUMC            | Area under the moment curve                                                                                 |
| AZT             | 3'-Azido-3'-deoxythymidine                                                                                  |
| AZTp2AZT        | Di(thymidine-3'-azido-2',3'-dideoxy- $\beta$ -D-ribose)-5',5'-p <sup>1</sup> ,p <sup>2</sup> -pyrophosphate |
| AZT-DP          | 3'-Azido-3'-deoxythymidine-5'-diphosphate                                                                   |
| AZT-DP-DSG      | 3'-Azido-3'-deoxythymidine diphosphate distearoylglycerol                                                   |
| AZT-MP          | 3'-Azido-3'-deoxythymidine-5'-monophosphate                                                                 |
| AZT-MP-DSG      | 3'-Azido-3'-deoxythymidine monophosphate distearoylglycerol                                                 |
| AZT-TP          | 3'-Azido-3'-deoxythymidine-5'-triphosphate                                                                  |
| AZT-TP-DSG      | 3'-Azido-3'-deoxythymidine triphosphate distearoylglycerol                                                  |
| BBB             | Blood-brain-barrier                                                                                         |
| br.             | Broad                                                                                                       |
| °C              | Degrees Celsius                                                                                             |
| C 7:0           | Heptanoic acid                                                                                              |
| C 10:0          | Decanoic acid                                                                                               |
| C 11:0          | Undecanoic acid                                                                                             |
| C 12:0          | Dodecanoic acid                                                                                             |
| C 13:0          | Tridecanoic acid                                                                                            |
| C 14:0          | Tetradecanoic acid (myristic acid)                                                                          |
| C 15:0          | Pentadecanoic acid                                                                                          |
| C 16:0          | Hexadecanoic acid                                                                                           |
| C 17:0          | Heptadecanoic acid                                                                                          |
| C 18:0          | Octadecanoic acid                                                                                           |
| CA              | Capsid protein                                                                                              |

|                      |                                                     |
|----------------------|-----------------------------------------------------|
| <i>C. albicans</i>   | <i>Candida albicans</i>                             |
| calcd                | Calculated                                          |
| CD4                  | Cluster of differentiation (class II MHC T cells)   |
| CDI                  | N,N'-Carbonyl dimidazole (1,1'-carbonyldiimidazole) |
| CDS                  | Chemical delivery system                            |
| CL                   | Total body clearance                                |
| cm                   | Centimeter                                          |
| Cmax                 | The peak or maximum concentration                   |
| <i>C. neoformans</i> | <i>Cryptococcus neoformans</i>                      |
| <sup>13</sup> C NMR  | Carbon nuclear magnetic resonance spectroscopy      |
| CNS                  | Central nervous system                              |
| CSF                  | Cerebrospinal fluid                                 |
| d                    | Doublet                                             |
| dATP                 | Deoxyadenosine triphosphate                         |
| dCTP                 | Deoxycytidine triphosphate                          |
| ddC                  | 2',3'-Dideoxycytidine                               |
| ddI                  | 2',3'-Dideoxyinosine                                |
| ddNs                 | 2',3'-Dideoxynucleosides                            |
| ddNTP                | 2',3'-Dideoxynucleoside triphosphate                |
| DEAD                 | Diethyl azodicarboxylate                            |
| dGTP                 | Deoxyguanosine triphosphate                         |
| DHBV                 | Duck hepatitis B virus                              |
| DIAD                 | Diisopropyl azodicarboxylate                        |
| DMAP                 | 4-Dimethylaminopyridine                             |
| DMF                  | N,N-Dimethyl formamide                              |
| DMSO                 | Dimethyl sulfoxide                                  |
| DNA                  | Deoxyribonucleic acid                               |
| dNTP                 | Deoxynucleoside triphosphate                        |
| ds                   | Double stranded                                     |
| D4T                  | 2',3'-Didehydro-3'-deoxythymidine                   |
| 3dT                  | 3'-Deoxythymidine                                   |
| 3dTDP                | 3'-Deoxythymidine diphosphate                       |
| 3dTDP-DMG            | 3'-Deoxythymidine diphosphate dimyristoylglycerol   |
| dTMP                 | Deoxythymidine-5'-monophosphate                     |
| dTTP                 | Deoxythymidine-5'-triphosphate                      |

|                      |                                                                                                                                                               |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| EC <sub>50</sub>     | EC <sub>50</sub> is defined as the 50% antiviral effective concentration, required to produce a 50% reduction in the cytopathic HIV effect in CD4 lymphocytes |
| EDTA                 | Ethylenediaminetetraacetic acid                                                                                                                               |
| e.g.                 | For example                                                                                                                                                   |
| EL                   | Ether lipid analogs                                                                                                                                           |
| ELSD                 | Evaporative light scattering detector                                                                                                                         |
| env                  | Envelope protein precursor gene                                                                                                                               |
| Env                  | Envelope protein precursor                                                                                                                                    |
| Eq                   | Equation                                                                                                                                                      |
| eq                   | Equivalent                                                                                                                                                    |
| eV                   | Electron volt                                                                                                                                                 |
| F                    | Fischer statistics                                                                                                                                            |
| FA(s)                | Fatty acid(s)                                                                                                                                                 |
| Fig.                 | Figure                                                                                                                                                        |
| FLT                  | 3'-Fluoro-3'-deoxythymidine                                                                                                                                   |
| FLTTP                | 3'-Fluoro-3'-deoxythymidine triphosphate                                                                                                                      |
| g                    | Gram(s)                                                                                                                                                       |
| g                    | Gravity                                                                                                                                                       |
| gag                  | Group antigen gene                                                                                                                                            |
| Gag                  | Group antigen                                                                                                                                                 |
| GAMT                 | 5'-O-Glucuronide-3'-amino-3'-deoxythymidine                                                                                                                   |
| GAZT                 | 5'-O-Glucuronide-3'-azido-3'-deoxythymidine                                                                                                                   |
| GGTSSLPA             | Glycine-Glycine-Threonine-Serine-Serine-Leucine-Proline-Alanine                                                                                               |
| GLA                  | Gammalinoleic acid                                                                                                                                            |
| GQHPALSM             | Glycine-Glutamine-Histidine-Proline-Alanine-Leucine-Serine-Methionine                                                                                         |
| Gly                  | Glycine                                                                                                                                                       |
| gp                   | Glycoprotein                                                                                                                                                  |
| h                    | Hour (s)                                                                                                                                                      |
| HBV                  | Hepatitis B virus                                                                                                                                             |
| <i>H. capsulatum</i> | <i>Histoplasma capsulatum</i>                                                                                                                                 |
| HIV                  | Human immunodeficiency virus                                                                                                                                  |
| <sup>1</sup> H NMR   | Proton nuclear magnetic resonance spectroscopy                                                                                                                |
| hNMT                 | Human NMT                                                                                                                                                     |

|                           |                                                                                                                                                                         |
|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| HPLC                      | High performance liquid chromatography                                                                                                                                  |
| HSV                       | Herpes simplex virus                                                                                                                                                    |
| Hz                        | Hertz                                                                                                                                                                   |
| IC <sub>50</sub>          | IC <sub>50</sub> is defined as the test compound concentration required to reduce the number of viable untreated CD4 lymphocytes by 50% or 50% inhibitory concentration |
| Ig                        | Immunoglobulin                                                                                                                                                          |
| ip                        | Intraperitoneal                                                                                                                                                         |
| IR                        | Infrared                                                                                                                                                                |
| iv                        | Intravenous                                                                                                                                                             |
| K                         | Equilibrium constant                                                                                                                                                    |
| K <sub>10</sub>           | The rate at which a drug leaves the system from the central compartment                                                                                                 |
| K <sub>12</sub>           | Transfer rate, 1 to 2                                                                                                                                                   |
| K <sub>21</sub>           | Transfer rate, 2 to 1                                                                                                                                                   |
| K <sub>10</sub> half-life | The half-life associated with the rate K <sub>10</sub>                                                                                                                  |
| K <sub>m</sub>            | Michaelis constant                                                                                                                                                      |
| L                         | Liter(s)                                                                                                                                                                |
| L-AC2                     | The diacylated L- $\alpha$ -phosphatidylcholine analog                                                                                                                  |
| Log D                     | Distribution coefficient                                                                                                                                                |
| Log P                     | Partition coefficient                                                                                                                                                   |
| m                         | Multiplet                                                                                                                                                               |
| M                         | Molar concentration                                                                                                                                                     |
| MA                        | Matrix protein                                                                                                                                                          |
| Mem                       | Minimum essential concentration                                                                                                                                         |
| mg                        | Milligram                                                                                                                                                               |
| mg/kg                     | Milligram per kilogram                                                                                                                                                  |
| MIC                       | Minimum inhibitory concentration                                                                                                                                        |
| <i>M. verrucaria</i>      | <i>Myrothecium verrucaria</i>                                                                                                                                           |
| min                       | Minute(s)                                                                                                                                                               |
| mL                        | Milliliter(s)                                                                                                                                                           |
| mM                        | Millimolar concentration                                                                                                                                                |
| MM+                       | The most general method for molecular mechanics calculations                                                                                                            |
| mmol                      | Millimole(s)                                                                                                                                                            |
| MOI                       | Ratio of number of infectious virus particles/number of target cells                                                                                                    |
| mol                       | Mole(s)                                                                                                                                                                 |



|                    |                                                                   |
|--------------------|-------------------------------------------------------------------|
| MOPS               | 3-[ <i>N</i> -morpholino]propane-sulfonic acid                    |
| mp                 | Melting point                                                     |
| MRT                | Mean residence time                                               |
| MS                 | Mass spectrometry                                                 |
| MW                 | Molecular weight                                                  |
| μ                  | Micro                                                             |
| μg                 | Microgram(s)                                                      |
| μL                 | Microliter(s)                                                     |
| μmol               | Micromole(s)                                                      |
| N                  | Normal                                                            |
| NAD (NADH)         | Nicotinamide adenine dinucleotide                                 |
| NADH               | Nicotinamide adenine dinucleotide, reduced                        |
| NC                 | Nucleic acid binding protein                                      |
| Nef                | Negative factor (Myristoylated cytoplasmic protein)               |
| NIH                | National Institutes of Health                                     |
| NLS                | Nuclear localization signals                                      |
| NMR                | Nuclear magnetic resonance spectrometry                           |
| NMT                | Myristoyl-CoA:protein <i>N</i> -myristoyltransferase              |
| NMT1p              | <i>S. cerevisiae</i> NMT                                          |
| P                  | Partition coefficient                                             |
| PD                 | Prodrug                                                           |
| PEG 200            | Polyethylene glycol 200                                           |
| pH                 | Log hydrogen ion concentration                                    |
| <i>pKa</i>         | Acidity constant                                                  |
| PM3                | A reparameterization of AM1 (a semiempirical method)              |
| <i>Pol</i>         | Polymerase gene                                                   |
| Pol                | Polymerase protein                                                |
| P(Ph) <sub>3</sub> | Triphenylphosphine                                                |
| ppm                | Parts per million (NMR)                                           |
| PR                 | Protease                                                          |
| PSIG               | Pounds per square inch gauge                                      |
| q                  | Quartet                                                           |
| QSARs              | Quantitative structure-activity relationships                     |
| quint              | Quintuplet                                                        |
| R <sup>2</sup>     | Multiple correlation coefficient                                  |
| <i>re</i>          | Relative brain exposure (AUC) <sub>pd</sub> →p/(AUC) <sub>p</sub> |

|                      |                                                                            |
|----------------------|----------------------------------------------------------------------------|
| Rev                  | Posttranscriptional regulator of viral gene expression                     |
| R <sub>f</sub>       | Retardation factor (rate of flow)                                          |
| RMS                  | Total energy gradient calculated as a root mean square value               |
| RMSD                 | Root mean square                                                           |
| RNA                  | Ribonucleic acid                                                           |
| RNase                | Ribonuclease                                                               |
| rpm                  | Revolutions per minute                                                     |
| RT                   | Reverse transcriptase                                                      |
| R.T.                 | Retention time                                                             |
| s                    | Second (s)                                                                 |
| s                    | Singlet                                                                    |
| SARs                 | Structure-activity relationships                                           |
| <i>S. cerevisiae</i> | <i>Saccharomyces cerevisiae</i>                                            |
| SD                   | Standard deviation                                                         |
| SDS                  | Sodium dodecyl sulfate                                                     |
| SEM                  | Standard error of the mean                                                 |
| SLPM                 | Standard liter per minute                                                  |
| S <sub>N</sub> 2     | Nucleophilic substitution bimolecular                                      |
| t                    | Triplet                                                                    |
| t <sub>1/2</sub>     | Half-life                                                                  |
| t <sub>1/2α</sub>    | Half-life of the distribution phase                                        |
| t <sub>1/2β</sub>    | Half-life of the terminal phase                                            |
| TAR                  | Trans-acting response sequence                                             |
| Tat                  | Transcriptional and posttranscriptional regulator of viral gene expression |
| 3-TC                 | 3'-Thia-2',3'-dideoxycytidine                                              |
| T-cell               | Thymus derived cell                                                        |
| Thd                  | Thymidine                                                                  |
| TK                   | Thymidine kinase                                                           |
| TLC                  | Thin layer chromatography                                                  |
| t <sub>max</sub>     | time to reach C <sub>max</sub>                                             |
| TS                   | Thymidylate synthase                                                       |
| UV                   | Ultraviolet                                                                |
| V <sub>m</sub>       | Maximum velocity                                                           |
| vs                   | Versus                                                                     |
| V <sub>ss</sub>      | Volume of distribution at steady state                                     |

WHO  
WienI

World Health Organization  
Wiener index (the first topological index)

## 1. INTRODUCTION

### 1.1. Acquired Immunodeficiency Syndrome (AIDS)

AIDS was first recognized as a new and distinct clinical entity in 1981 (Gottlieb *et al.*, 1981, Masur *et al.*, 1981). Essex *et al.* (1983), Gallo *et al.* (1983), and Montagnier and his colleagues (Barré-Sinoussi *et al.*, 1983) postulated that a variant T-lymphotropic retrovirus (HTLV) could be the etiologic agent of AIDS. Among the most compelling reasons for this hypothesis was that HTLV, discovered by Gallo and colleagues in 1980 (Poiesz *et al.*, 1980), was the only human virus known to infect T-helper lymphocytes. Further characterization of the agent, now termed human immunodeficiency virus type 1 (HIV-1), revealed that it was only distantly related to HTLV yet was the same as the isolate detected earlier by Montagnier and his colleagues (Barré-Sinoussi *et al.*, 1983). HIV is a member of the lentivirus family of retroviruses. The epidemic of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) has affected over 190 countries within less than two decades. By the year 2000, the World Health Organization (WHO) currently projects a cumulative total close to 40 million HIV infections in men, women and children. The projected cumulative total of adult AIDS cases for the year 2000 is well over 10 million (Merson, 1993). HIV infection can be transmitted in three ways: through sexual intercourse, through blood, and from mother to child (vertical transmission) (World Health Organization, 1993).

#### 1.1.1. The composition of the HIV particle

HIV exhibits a characteristic cone-shaped core that is surrounded by a bilayered lipid envelope derived from the host cell membrane. This envelope is studded by characteristic knobs that represent oligomeric structures of the virally encoded envelope glycoproteins gp120 and gp41. The gp120 subunit comprises the extracellular portion of the viral envelope, and the gp41 portion spans the membrane and anchors the glycoprotein complex to the surface of virions. The two glycoproteins are noncovalently linked and thus form fragile structures that often dissociate. Below the lipid bilayer is the matrix protein p17 (MA), which lines the inner surface of the virus particle and interacts with the membrane via a myristic acid moiety. The inner cone-shaped core is comprised of the

major capsid protein p24 (CA), which surrounds two copies of the viral RNA bound to the nucleic acid binding protein p9 (NC). The capsid structure also contains viral *pol* gene products, including the protease, reverse transcriptase, and integrase (Figure 1.1) (Hahn, 1994).

The human immunodeficiency virus (HIV) shares features common to all retroviruses. First, retroviruses are so named because of their ability to route genetic information from RNA to DNA. This is accomplished by a unique enzyme, reverse transcriptase (RT), which is encoded by a gene within the retroviral genome. All retroviruses have in common the three coding regions *gag*, *pol*, and *env*, which encode the capsid proteins (Gag), the viral enzymes necessary for replication (Pol), and the external glycoprotein (Env) that protrudes out of the lipid viral envelope and is responsible for the infectivity of the viral particle by means of attachment to specific cellular receptors. The viral enzymes encoded by *pol* are reverse transcriptase, integrase and protease. The additional proteins expressed by HIV-1 (e.g., Tat, Rev) directly regulate viral gene expression (Hahn 1994).

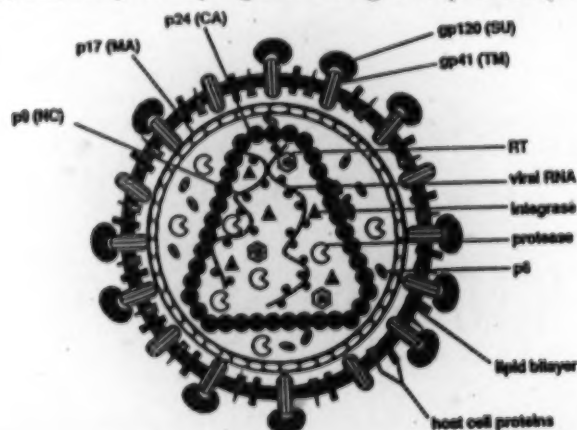


Figure 1.1. Schematic representation of the HIV particle (Hahn, 1994).

#### 1.1.2. Markers of disease progression (CD4, HIV-1 RNA)

The primary targets of HIV infection are the immune system and the nervous system. One of the most fortuitous finding in AIDS research was that the molecule used to identify the cell type (CD4), which has been observed to be depleted during disease progression, was also the major receptor for HIV-1 (Dalglish *et al.*, 1982). CD4, a member of the immunoglobulin (Ig) superfamily, is expressed on the surface of helper T lymphocytes and

participates in normal class II major histocompatibility complex (MHC) recognition in association with T-cell responsiveness to foreign antigens (Callebaut *et al.*, 1993). CD4 is found in T-lymphocytes, monocytes, B lymphocytes, macrophages and other cells and is also the receptor for HIV-2 and simian immunodeficiency virus (SIV). The use of CD4 cell counts represents the best means of following the natural history of HIV disease. For example, all patients with CD4 counts less than 200 cells/mm<sup>3</sup> are considered as having AIDS (Hoover *et al.*, 1992). The HIV infection of CD4 lymphocytes results in cytolysis and cell death, both of which contribute to the immunodeficiency of AIDS; however, CD4 monocytes and macrophages may not be greatly harmed by the virus. Viral replication in these cells appears to be more prolonged and less cytotoxic than in lymphocytes, and as a result, monocyte and macrophages represent important reservoirs of HIV-1 with an important role in the pathogenesis of AIDS. HIV is far from being inactive during the asymptomatic part of the infection but replicates at a fast rate in lymph nodes and peripheral blood cells. One of the major advances in recent AIDS research has been the advent and implementation of techniques to accurately measure HIV-1 RNA in plasma (Mellors *et al.*, 1996, O'Brien *et al.*, 1996). The risk of disease progression (AIDS or death) appears to be directly related to the levels of plasma HIV-1 RNA, referred to as plasma viral load. This marker is a better predictor of outcome than numbers of peripheral blood CD4 cells. The measurement of HIV RNA better meets the criteria for an ideal surrogate marker than any other candidate markers for the treatment effects of several anti-HIV therapies (Mellors *et al.*, 1996, O'Brien *et al.*, 1996, Saag *et al.*, 1996). High turnover of HIV in blood has been revealed by new studies. Billions of virus particles are continuously produced by newly infected cells that are rapidly cleared. It is believed that HIV does not mutate more frequently than other RNA viruses, and drug resistance is a consequence of the high level of replication (Ho *et al.*, 1995).

### 1.2. Clinical manifestation of AIDS

Infection with HIV-1 induces an insidious, progressive loss of immune system function, which ultimately results in the opportunistic infections and malignancies associated with acquired immunodeficiency syndrome (AIDS). Among the diseases which



are infrequently observed in immunologically normal individuals is Kaposi's sarcoma, a rare form of skin cancer, and numerous opportunistic infections such as *Pneumocystis carinii* pneumonia, toxoplasmosis, histoplasmosis, cryptococcosis, and cytomegalovirus. The median time from initial infection to the development of AIDS is approximately 10 years, although the rate of disease progression varies substantially. AIDS-related complex (ARC) (unexplained fevers, night sweats, chronic diarrhea, and wasting) is defined as a complication of symptoms and opportunistic processes that, although not constituting an AIDS-defining condition, do result from immune dysfunction caused by HIV-1 infection (Schnittman *et al.*, 1997). Some important clinical manifestations of infection with HIV, related to this thesis project and the problems in their treatment, are described briefly. Various strategies used in this project against these infections are discussed later.

#### 1.2.1. Fungal infections in patients with AIDS

Fungal infections are common complications of infection with HIV. *C. neoformans* is the major cause of meningitis in AIDS patients, and it can also cause local organ dysfunction and disseminated disease. *C. neoformans* is a yeast-like fungus that reproduces by budding. It produces no known toxins, but it does have a large polysaccharide capsule that appears to play a role in protecting the organism from host defense mechanisms. It is the only encapsulated yeast known to be pathogenic in humans, and it is the most common cause of fungal meningitis. Infection occurs via inhalation of spores. Depending on the infecting dose and immunologic status of the patient, chronic systemic infection affecting the lungs, skin, or meninges can occur. The most common site of infection with *C. neoformans* is the central nervous system (CNS). In immunocompromised patients, such as those with AIDS, cryptococcosis follows a more malignant course, frequently involving bizarre cutaneous manifestations and spreading to the central nervous system, where it is often refractory to treatment (Diamond, 1995).

*Candida* are fungi that exist predominantly in unicellular forms and reproduce by budding. Species of the genus *Candida*, which are part of the normal human flora, are the most common yeast pathogens. *Candida albicans* is the most frequently identified pathogen among these species. *Candida albicans* is a dimorphic, asexual yeast that is a

major cause of systemic fungal infection in immunocompromised humans. Oral candidiasis is a marker of an impaired immune system and a prognostic marker for the subsequent development of opportunistic infections (Klein *et al.*, 1984).

*Aspergillus* is a common mold found in the soil that frequently causes invasive pulmonary disease in immunocompromised patients. Pulmonary aspergillosis predominately occurs as a complication of AIDS. Both invasive pulmonary and obstructing bronchial aspergillosis have been described in patients with AIDS (Lortholary *et al.*, 1993).

#### 1.2.1.1. Common antifungal agents

At the present time, therapy for systemic *C. neoformans* and *C. albicans* infections is less than ideal. Patients are currently treated with amphotericin B (1) or lanosterol 14 $\alpha$ -demethylase inhibitors such as the imidazole analog ketoconazole (2) or the triazole fluconazole (3) (Figure 1.2). Amphotericin B (1) is a polyene antibiotic which binds to the fungal sterol ergosterol in the cell membrane and promotes the loss of vital ions from the cell (Figure 1.3). Amphotericin B is fungicidal and has a higher affinity for ergosterol than its mammalian counterpart cholesterol, and is thus less toxic to mammalian cells. Cryptococcosis in AIDS patients, unlike that in immunocompetent hosts, is frequently resistant to treatment with amphotericin B. Moreover, while amphotericin B (1) is the only fungicidal agent available, it is nephrotoxic, does not penetrate into the cerebrospinal fluid, and must be given intravenously. However, amphotericin also has some affinity for the mammalian sterol cholesterol, and this lack of selectivity results in a number of side effects in man, including shaking, chills, fever, cramps, nausea, vomiting and renal failure. These side effects, together with the fact that amphotericin has to be given by slow intravenous infusion, have made clinicians reluctant to use it, and frequently amphotericin has been used as a last resort, when it is too late.

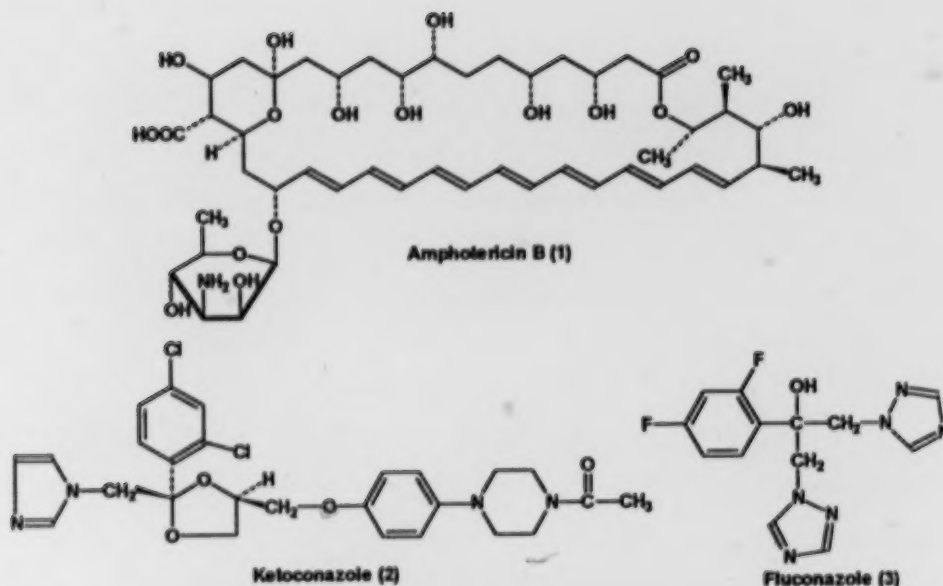


Figure 1.2. Structures of some antifungal agents.

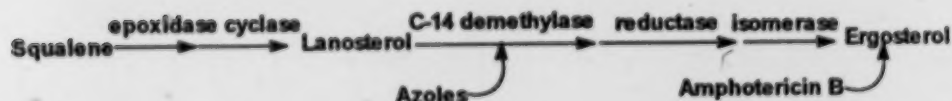


Figure 1.3. Ergosterol synthetic pathway, showing sites of inhibition by different antifungal agents

The azole derivatives, discovered in the late 1960s, are totally synthetic and are the most rapidly expanding group of antifungal compounds. They are classified as imidazoles or triazoles. Depending on the particular compound, azole antifungal agents exhibit broad-spectrum fungistatic activity that includes most yeasts and filamentous fungi. They act primarily on ergosterol biosynthesis at the C-14 demethylation stage, a three-step oxidative reaction catalyzed by the cytochrome P-450 enzyme 14 $\alpha$ -sterol demethylase (Figure 1.3) (Richardson *et al.*, 1993, Georgopapadakou N. H. *et al.*, 1996). The imidazole and triazole antifungals have also been known to be very effective clinically. Ketoconazole is metabolically less vulnerable than earlier imidazole antifungal derivatives,

resulting in good oral bioavailability. Ketoconazole (2) and the newer azoles are fungistatic rather than fungicidal. Fluconazole (3) is effective against many of the causes of systemic fungal infections in patients with AIDS, but chronic and prophylactic therapy has been associated with the development of resistant organisms. For the treatment of oropharyngeal candidiasis in AIDS patients, fluconazole has a 100% cure rate (Richardson *et al.*, 1993).

Response to treatment against aspergillosis has been poor. Intravenous amphotericin B has been used with limited success (Lortholary *et al.*, 1993).

### 1.2.2. Liver disease and AIDS

The liver can be directly infected by the HIV virus. Both hepatic macrophages, including Kupffer cells and the sinusoidal endothelial cells, express CD4 which mediates uptake by cells (Scazec, 1990). *C. neoformans* and *C. albicans* may infect the liver, usually in association with systemic disease (Glasgow *et al.*, 1985). Coinfection with other viruses may alter the progression of HIV infection.

#### 1.2.2.1. Hepatitis viral infections in human immunodeficiency virus-infected individuals

Five percent of the world's population, over 300 million people, are chronically infected with hepatitis B virus (HBV). HBV replicates largely in the liver. The mechanism by which the virus enters the hepatocyte is unclear since no specific receptors for HBV on hepatocytes have been identified. Hepatitis B virus infections cause severe diseases such as acute hepatitis (which causes abdominal pain, jaundice, and elevated levels of certain enzymes), fulminant hepatitis (a fatal form of the disease in which massive sections of the liver are destroyed) and chronic hepatitis (which can cause fatigue, cirrhosis of the liver, and hepatocellular carcinoma). It is estimated that 5000 people die from HBV related cirrhosis each year in the USA. Most individuals infected with HIV are also infected with at least one other virus. HIV replicates in CD4<sup>+</sup> cells, macrophages, microglial cells of the central nervous system, gut epithelial cells, and hepatic endothelial cells. Replication of HBV occurs primarily in hepatocytes and, to a small extent, in lymphocytes (Roisman *et*

*al.*, 1994, Scoazec *et al.*, 1990). However both viruses are present and available for transmission by blood and genital tract secretions. HBV and HIV are also spread to offspring by exposure of infected maternal fluids (ie, blood, vaginal secretions, and in the case of HIV by breast milk), or by *in utero* passage of the virus across the placenta to the fetus (Hilleman *et al.*, 1994). Because these viruses are transmitted in similar fashions, it is not surprising that most HIV-seropositive persons are also infected with HBV (Hilleman *et al.*, 1994). Troisi *et al.* (1993) reported that almost 90% of HIV-seropositive men showed evidence of HBV infection. HBV infection was also identified by Greenspan and colleagues (1991) as a risk factor for progression to AIDS. Although, there are distinct differences between HBV and HIV infections, HBV infection has been suggested as a potential cofactor for HIV infection (Twu *et al.*, 1993). One notable similarity between these two human pathogenic viruses is that their replicative cycles involve an obligate RNA intermediate and a reverse transcription step in the cytoplasm (Figures 1.4 and 1.6). HBV contains a specific DNA polymerase required for vital replication. This DNA polymerase also acts as a reverse transcriptase during the replication of HBV DNA via an RNA intermediate (Locarnini *et al.*, 1996).

#### 1.2.2.2. Composition and life cycle of the HBV particle

Hepatitis B is induced by a 42-nm double-shelled DNA virus that is the prototype member of the family of viruses known as hepadnaviruses. The virus has an outer envelope component of hepatitis B surface antigen (HBsAg) and an inner nucleocapsid component of hepatitis B core antigen (HBcAg). Within the nucleocapsid core, there is endogenous DNA polymerase activity with a single molecule of partially double-stranded circular DNA (Locarnini *et al.*, 1996).

The replication of hepadnaviruses differs markedly from that of other DNA viruses. A major difference is a reverse transcription step analogous to that seen in retroviruses. (1) After the hepadnavirus penetrates the target cells (hepatocytes), the virus is uncoated and the DNA enters the nucleus. (2) In the nucleus, the partially double stranded (ds) DNA of the virus is converted to a double stranded covalently closed circular form (supercoiled DNA) by DNA polymerase activity and a ligase. (3) The supercoiled DNA is transcribed



into major RNA forms (2.1 kb and 3.5 kb). The pregenomic RNA (3.5 kb) is synthesized by cellular RNA polymerase. (4) The pregenomic RNA can either be transcribed into viral proteins (HBsAg, HBcAg, DNA polymerase), or (5) it can be packaged into core particles and (6) reverse transcribed into minus-strand DNA. (7) The minus-strand DNA serves as a template for plus-strand DNA which is accomplished by the viral DNA polymerase (Molnar-Kimber *et al.*, 1984, Will *et al.*, 1987, Locarnini *et al.*, 1996). (8) The synthesis of the plus-strand may not be completed before coating by nucleocapsids and export of viral particles (Locarnini *et al.*, 1996) (Figure 1.4).

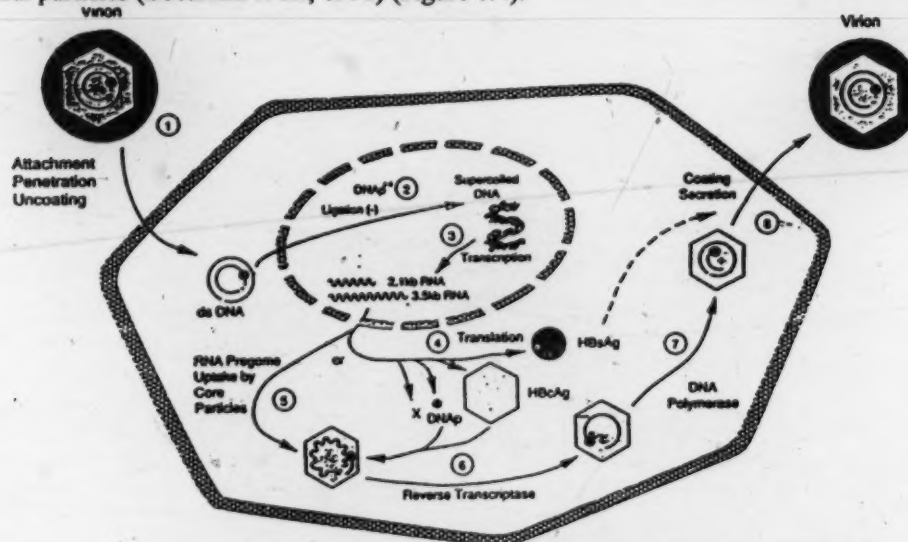


Figure 1.4. The replicative cycle of HBV (Hoopnagle *et al.*, 1990).

#### 1.2.2.3. Available therapies against HBV

Vaccination has been used for the prevention of HBV infection. However, at the present time, there are no clinically effective drugs available for the treatment of HBV infection (Harmenberg *et al.*, 1996). Interferon-alpha has been shown to be therapeutically useful in the treatment of hepatitis B. However, only a minority of patients respond to interferon. Furthermore, treatment by subcutaneous injection must continue for months, and toxicity at effective doses is substantial. Suppression of levels of HBV DNA or viral DNA polymerase may take weeks of interferon-alpha therapy to be apparent (Alexander *et al.*, 1987). A variety of compounds have been synthesized or tested by different groups to



develop a treatment against HBV infection (Hostetler *et al.* 1993a, Matthes *et al.*, 1992, Tyrrell *et al.*, 1991, Adair *et al.*, 1995, Chu *et al.*, 1996). Recently, 2',3'-dideoxy-3'-thiacytidine (3TC) (4) (Soudeyns *et al.*, 1991) and 2',3'-dideoxycytidine (ddC) (5) (Mitsuya, *et al.*, 1990) have been shown to be potent inhibitors of replication of both human immunodeficiency virus and hepatitis B virus (Yokota *et al.*, 1991) *in vitro* (Figure 1.5). However, long-term ddC usage causes delayed toxicity such as peripheral neuropathy in patients, which was suggested to be a result of the depletion of mitochondrial DNA in host cells treated with ddC (Chen *et al.*, 1989). 3TC (4) has been subjected to clinical trials in HBV-infected patients (Doong *et al.*, 1991). In light of the fact that HBV causes severe and often tragic effects in the infected patient, there remains an urgent need to develop new effective pharmaceutical agents that have low toxicity to the host.

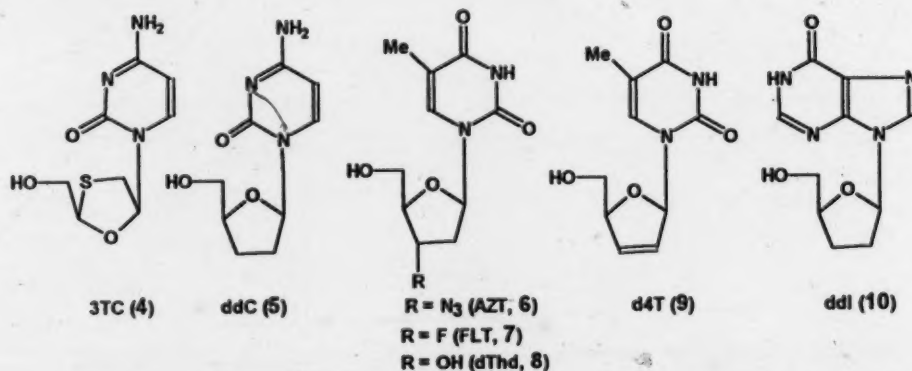


Figure 1.5. The chemical structures of 3TC (4), ddC (5), AZT (6), FLT (7), dThd (8), d4T (9) and ddi (10).

### 1.2.3. Central nervous system complications

Human immunodeficiency virus (HIV) also invades the central nervous system (CNS), ultimately culminating in severe neurological disorders and increased susceptibility to persistent infections due to suppression of the immune system. A variety of infections of the CNS commonly complicate late stage HIV-1 infection, including most importantly, *C. neoformans*. Cryptococcal meningitis is the most common CNS fungal infection in AIDS, eventually affecting 5 to 10% of patients (Diamond, 1995). One of the most destructive

aspects of AIDS is an encephalopathy associated with direct infection of the central nervous system (CNS) by HIV. Brain macrophages, microglia and multinucleated cells can be productively infected with HIV-1. The mechanism by which the virus crosses the blood brain barrier (BBB) has not been specifically defined, but it is believed to result from migration of HIV-infected macrophages/monocytes into the brain. HIV can replicate in the brain and the infected brain serves as a reservoir for the virus (Koenig *et al.*, 1986). The neurologic complications of infection with HIV are both common and varied, and they contribute significantly to patient morbidity and mortality. Disorders of the CNS can complicate all stages of systemic HIV-1 infection, from the period after initial infection through the end stage of severe immunosuppression (Price, 1995). AIDS dementia complex (ADC), which is characterized by cognitive, motor, and behavioral dysfunction, usually develops later in the course of HIV-1 infection and is one of the most common late CNS complications of HIV disease. Accumulating evidence indicates that ADC can be treated and perhaps prevented, at least to some extent, by antiretroviral drug therapy. Zidovudine improves neuropsychologic performance in adults patients with HIV-1 infection, compared to placebo. Patients receiving AZT (6) showed a general improvement of neurological functions including attention, memory, motor function, and general cognitive ability (Sidtis *et al.*, 1993).

#### **1.2.3.1. Drug delivery to brain**

Since the infected brain may serve as a sanctuary for the HIV virus, from which the periphery may continuously be reinfected, it is essential that the anti HIV agent crosses the BBB readily to deliver an effective therapeutic concentration in brain without increasing toxicity. The design of anti-HIV drugs that penetrate to CNS and provide a therapeutic brain concentration has presented a significant challenge to medicinal chemists since the brain is a reservoir for HIV.

#### **1.2.3.2. Blood brain barrier (BBB)**

The term blood-brain barrier (BBB) is commonly defined as the anatomical and physiological complex that controls the movement of substances from the general

extracellular fluid of the body into the brain (Bodor *et al.*, 1983). The endothelial cells in cerebral capillaries are tightly joined in comparison with other organs such as intestine, kidney and salivary gland. In addition the pinocytotic vesicles are almost absent in brain capillary endothelium. These tight junctions, absence of fenestrations, and relative lack of pinocytosis severely restrict the nonspecific transport of materials into the brain (Bodor *et al.*, 1983, 1987). However, it appears that the drug delivery to brain is affected not only by the structure of cerebral capillaries, but by other factors such as the lower vesicular content, osmosis, ultrafiltration, net charge and/or lipid solubility of the compound, and affinity for transport carrier systems which restrict the entry of some materials and facilitate the entry of others into the brain. Lipophilic compounds can readily pass through these phospholipoidal membranes, but most hydrophilic compounds and high molecular weight substances are excluded (Olendorf 1974, 1978, Levin 1980, Shah *et al.* 1989). The efflux of compounds from the brain also varies. For example, Wang *et al.* (1995) suggested that the transport of AZT (6) across the BBB is asymmetric since the efflux clearance was five times greater than the influx clearance in rabbit, indicating the existence of active transport processes for the transport of AZT (6) from brain to plasma across the blood-brain barrier (BBB) (Wang *et al.*, 1995). These data suggest that delivery of high AZT (6) levels may saturate the active elimination mechanism, thereby providing for slower brain efflux and higher than expected AZT (6) concentrations (Brewster *et al.*, 1995).

Three major factors are involved in the passage process of drugs into the CNS: (1) drug lipophilicity and ionization, (2) molecular size, (3) plasma protein binding.

**(1) Drug lipophilicity and ionization:** The relative distribution between water and lipid, as expressed by its *n*-octanol/water partition coefficient, determines the ease by which a molecule is able to permeate the lipid membranes of the endothelial cells. If a drug is ionized at physiological pH, its effective lipophilicity is substantially lower, because the ionized species are surrounded by water molecules due to ion-dipole interaction that disfavors membrane interaction (van Bree *et al.*, 1992, 1993). AZT (6) is one of the more lipophilic (Log P = 0.11) anti-HIV compounds investigated clinically, and it readily enters the cerebrospinal fluid (CSF) possibly by both passive diffusion and active transport.

However, the entry of AZT (6) into brain tissue from the CSF is likely not sufficient to provide a therapeutic concentration that suppresses viral replication in the brain. Less than 1% of AZT (6) injected via the carotoid artery in rats penetrated into brain (Ellison *et al.*, 1988). AZT (6) has been reported to enter cells either predominantly or partially by nonfacilitated diffusion due to its relatively high lipophilicity ( $\text{Log } P = 0.11$ ).

Olendorf determined the passage of a large number of substances across the BBB and found a sigmoidal relationship between the olive oil/water partition coefficient and concentration in brain, suggesting the existence of a threshold lipophilicity (Olendorf *et al.*, 1974). When a more sensitive method was used, an empirical relationship was found between the Log value of blood-brain barrier permeability (corrected for differences in protein binding and diffusion coefficient) and the Log value of the *n*-octanol/water partition coefficient. A linear equation gave the best description of the data (Rapoport *et al.*, 1979). Recent studies showed that penetration of drugs into the brain does not correlate with  $\text{Log } P_{\text{octanol}}$  values. Indeed, it was found for  $\text{H}_2$ -antagonists that the difference between partitioning in *n*-octanol/water and cyclohexane/water ( $\Delta \text{Log } P = \text{Log } P_{\text{oct}} - \text{Log } P_{\text{chex}}$ ) correlates well with brain uptake, while there was a poor correlation with only one of these Log *P* values (Young *et al.*, 1988, El Taylor *et al.*, 1992, De Waterbeemd *et al.*, 1993).

(2) **Molecular size:** Molecular size is an important determinant of diffusion rates for drug molecules. Theoretical considerations imply a linear relationship between permeability and square root of molecular weight (MW), which could be verified by experimental data. This relationship is theoretically restricted to molecules with  $\text{MW} \leq 1,000$  (Levin *et al.*, 1980). Since the BBB has molecular sieving characteristics, probably due to sparse intercellular junctions, the passage of large hydrophilic molecules into the central nervous system is restricted (van Bree *et al.*, 14, 1992).

(3) **Plasma protein binding:** In order to have a perfect profile for penetration into the central nervous system, a drug should be a small lipophilic molecule with low protein binding (Pardridge, 1979).

Generally, a compound gains access to the brain via several routes. If the molecule has affinity for one of the carrier systems, it may be transported across the BBB by association



with this carrier. A compound with high intrinsic lipophilicity can diffuse passively through the phospholipoidal cell membrane matrix (Bodor *et al.*, 1983, Olendorf 1974, 1978).

#### **1.2.3.3. Chemical (pharmacological-based) approaches to improve delivery of drugs into the CNS**

Chemically-based drug delivery strategies involve the development of lipid-soluble prodrugs, and the site-specific chemical delivery system prodrugs (CDS) (Pardridge, 1989, Brewster, 1989, Bodor, 1987). Prodrugs have been designed which can be transported into the central nervous system because of their increased lipophilicity or affinity for one of the carrier systems present at the blood-brain barrier. After delivery, the pro-moiety is enzymatically cleaved and the active compound is released. This approach will be discussed in details in section 1.5.

#### **1.2.3.4. Antiviral agents for the treatment of HIV-1 brain infection**

Antiviral agents must gain access to the central nervous system (CNS) in a therapeutically relevant concentration if AIDS dementia is to be adequately treated. Pharmacokinetic considerations are often not met because of the ostensibly protective blood-brain barrier (BBB) which effectively prevents or reduces the entry of many hydrophilic drugs, including antiviral ribosides. In fact, uptake of AZT (6) may be BBB mediated. The low brain concentrations observed are not necessarily due to poor penetration, but rather to active AZT (6) loss from the brain. Sawchuck and colleagues (1990) found that elimination of AZT (6) from brain and blood was reduced by probencid, an inhibitor of weak acid transport at the level of the CSF and kidneys. For example, microdialysis studies have shown that probencid increases the AUC for AZT (6) in brain (thalamic extracellular fluid) by five- to six-fold. Dykstra *et al.* (1993) reported similar findings in rats where the limited uptake of AZT (6) is related to its efficient elimination from brain rather than to its low uptake into brain (Brewster *et al.*, 1997).

### **1.3. The life cycle of HIV-1**

The most important reason for studying the HIV viral life cycle is to identify

vulnerable steps that a potential therapeutic intervention may successfully interrupt. The steps in viral replication include: attachment or absorption, penetration, uncoating, reverse transcription, RNaseH degradation, DNA synthesis of second strand, migration to nucleus, integration, transcription or RNA processing, protein synthesis (translation), protein glycosylation, myristoylation, assembly of virus, release of virus, and maturation (Stevenson *et al.*, 1992, Greene *et al.*, 1993). The replication of HIV is illustrated in Figure 1.6.

**(1) Attachment and penetration:** The binding of an extracellular infectious virion to a susceptible cell initiates the afferent portion of the HIV retroviral life cycle. Binding occurs between the surface gp120 Env protein of the virus and the cellular CD4 molecule. This interaction permits the transmembrane gp41 Env to fuse the viral lipid envelope to the cell membrane. **(2) Uncoating:** Cytoplasmic penetration by the virus core results upon fusion, liberating the viral genomic RNA as a nucleoprotein complex. The nucleoprotein complex consists of the genomic RNA, the nucleocapsid (NC) and matrix (MA) proteins, and the viral enzymes reverse transcriptase and integrase. **(3) Reverse transcription:** Reverse transcriptase possesses three enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease H (RNase H). Reverse transcriptase uses RNA primers with 3' OH ends to initiate DNA synthesis. The RNase H activity is responsible for the cleavage of the RNA strand in the RNA-DNA hybrid. The RNA is then rapidly converted by RT into a copy of double-stranded DNA and transported to the nucleus. **(4) Integration:** Here, in association with viral integrase, the viral DNA is integrated by cleavage steps into host chromosomal DNA. This starts the efferent portion of the life cycle. The proviral DNA now functions as a mammalian gene and can replicate synchronously with host chromosomal genes. **(5) Transcription:** Expression of viral genes begins when the proviral DNA serves as template for the production of viral RNAs that include both genomic RNA molecules as well as mRNAs for viral protein synthesis that can be divided into early and late steps. **(6) Translation:** In the early steps of protein synthesis, multiply-spliced RNA produces the regulatory proteins (Tat, Rev, and Nef) that enhance and stabilize the later-stage mRNAs responsible for structural function. Accumulation of the Rev protein then causes a switch to the



production of unspliced and incompletely spliced RNA transcripts and the synthesis of structural proteins (Gag, Gag/Pol, and Env). These structural proteins assemble around viral genomic RNA at the plasma membrane. Gag and Gag/Pol precursor molecules are transported to the cell membrane via the cytoplasmic pathway. (7) **Protein glycosylation:** Precursor gp160 Env undergoes glycosylation and oligomerization in the endoplasmic reticulum. From this point, it is transported to the Golgi complex, where cellular enzymes process glycoprotein to surface gp120 and transmembrane gp41. Here, they associate noncovalently and are transported into the cell surface. (8) **Myristoylation:** Critical to final budding is myristoylation of the *N*-terminal glycine of the p17 MA protein by Myristoyl-CoA:protein *N*-myristoyltransferase (NMT). This event culminates the process of condensing the inner core shell and linking it with the glycoprotein-incorporated lipid membrane (Folks *et al.*, 1997). (9) **Release of virus and maturation:** The final step consists of proteolytic cleavage of Gag precursor molecules which occurs as the particle buds free from the cell, incorporating the Env protein and a portion of the plasma membrane into its outer envelope coat. As a final step in the HIV life cycle, the infectious virus matures after it has been released from the plasma membrane. This occurs as a coordinated process by which the viral protease (located between the Gag and Pol domains) cleaves the Gag-Pol protein in an ordered fashion. As the Gag and Gag-Pol proteins begin to encapsidate the HIV genomic RNA, the plasma membrane also begins to form a lipid bilayer around the viral core (Pavlakakis, 1997) (Figure 1.6).

### 1.3.1. Strategies and progress in the treatment of HIV infection

The development of effective antiviral therapies for the treatment of individuals infected with the human immunodeficiency virus (HIV) presents a unique challenge. The integration of the virus into the host cell DNA, the dependence of the virus on host cell machinery, the high mutation rate of HIV reverse transcriptase, and the chronicity of viral replication represent formidable obstacles to the development of specific and effective antiretroviral therapies.

Several review articles have discussed different strategies for HIV chemotherapy (Mitsuya *et al.*, 1990, Yarchoan *et al.*, 1991, Schinazi *et al.*, 1992b, De Clercq *et al.*,

1992, Sandström *et al.*, 1993a, b, Mohan P., 1993, De Clercq *et al.*, 1995, Aggarwal, *et al.*, 1990) (Figure 1.6).

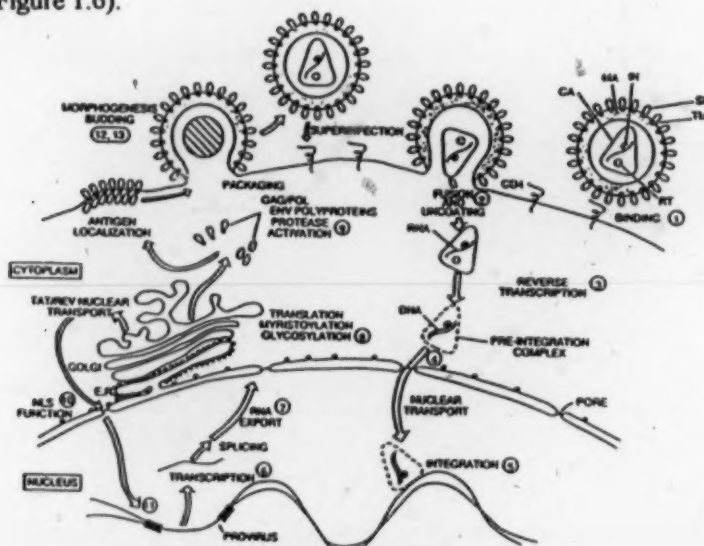


Figure 1.6. Stages of HIV-1 replication and agents for intervention. Numbers in the figure indicate the stage of HIV-1 replication to which the following agents may be targeted. (1) Low-molecular weight sulfated polysaccharides; soluble CD4; antibodies to HIV or host cell receptors; CD4 conjugated exotoxin. (2) Antibodies to secondary host cell receptors important in V3-dependent fusion. (3) Reverse transcriptase inhibitors-dideoxynucleoside analogs; TIBO compounds; RNase H inhibitors. (4) Inhibitors of nuclear transport of HIV-1 DNA; interruption of pre-integration complex translocation across nuclear pore. (5) Inhibitors of integrase function. (6) Antisense RNA; destruction of HIV-1 RNA by ribozymes; sense TAR analogs. (7) Inhibitors of function required for RNA export to site of packaging; RNA packaging sequence analogs. (8) Glucosidase inhibitors, e.g., castanospermine, and NMT inhibitors, e.g., 12-methoxydodecanoic acid. (9) Peptidomimetic protease inhibitors. (10) Peptidomimetic NLS motifs for inhibition of active *tat/rev* transport. Transdominant *tat/rev* mutants. Inhibitory heterologous virus transacting elements (e.g., AAV *rep* protein). (12) Interferon and synergistic compounds (e.g., ampliten). (13) Hypericins (Stevenson *et al.*, 1992).

There are several approaches to antiviral treatment such as virus adsorption inhibitors (polyanionic substances), virus-cell fusion inhibitors, nucleoside and nonnucleoside reverse transcriptase inhibitors (Sandström *et al.*, 1993b, De Clercq *et al.*, 1995, Morin *et al.*, 1997, Cantrell *et al.*, 1996), integrase inhibitors, Tat inhibitors, protease or proteinase inhibitors, myristoylation and glycosylation inhibitors, antisense oligonucleotides,

ribozymes, gene therapy and immunologic approaches (Schnittman *et al.*, 1997, Hirsch *et al.*, 1996). However, global efforts in the search for potential anti-HIV-1 agents have mainly centered around the design of enzyme inhibitors (reverse transcriptase, integrase, protease).

Several integrase inhibitors have been identified that include nucleotides, topoisomerase inhibitors, intercalators (Fesen *et al.*, 1993), aurintricarboxylic acids (Cushman *et al.*, 1992) and nucleotides.

Three protease inhibitors (Sanquinavir, Ritonavir, Indinavir) have been approved in the United States. These compounds are peptidomimetics that share common properties including a potent *in vitro* inhibitory effect on HIV-1 replication in acutely and chronically infected cells. Some limitations include complex synthetic processes, consequent drug availability, high protein binding, and the development of resistance *in vitro* or *in vivo* with preliminary evidence of cross-resistance. Protease inhibitors are synergistic with RT inhibitors, and consequently, multitarget combination studies are being conducted (Markowitz *et al.*, 1995).

#### 1.4. Targets used in this project for chemotherapeutic intervention

There are various specific events in the replicative cycle of human immunodeficiency virus (HIV) and hepatitis B virus (HBV), yeast (*Saccharomyces cerevisiae*) and fungi (*Candida albicans*, *Aspergillus niger*, and *Cryptococcus neoformans*) that can be considered as targets for chemotherapeutic intervention. In designing analogs, we considered two specific agents designed to act on two different targets simultaneously. These include, (1) reverse transcriptase inhibitors of the viral RNA to proviral DNA (HIV and HBV), and (2) fatty acids which exhibit their antiviral and antifungal activity by different mechanisms. One possible mechanism is inhibiting myristoyl-CoA:protein *N*-myristoyltransferase (NMT) that catalyzes the post-translational processing of viral and fungal proteins due to myristoylation.

#### 1.4.1. Reverse transcriptase inhibitors of viral RNA to proviral DNA in HIV and HBV.

Most efforts have concentrated on inhibitors of viral reverse transcriptase (RT), a key enzyme in the replicative cycle of the viruses (HIV, HBV) which is an attractive target for chemotherapy.

One cannot ignore the fact that reverse transcription comprises three different catalytic steps: (1) the conversion of (+)RNA to (-)DNA by RNA-dependent DNA polymerase, (2) the conversion of (-)DNA.(+)RNA to (-)DNA by ribonuclease H, and (3) the conversion of (-)DNA to (+)DNA.(-)DNA by DNA-dependent DNA polymerase. The RT reaction is a complex enzymatic reaction in that it requires four substrates (dATP, dGTP, dCTP, and dTTP), a primer, a template, and a number of cofactors (De Clercq, 1992). It is of interest that the life cycle of hepatitis B virus (HBV) also involves the enzyme reverse transcriptase in its DNA replication (Will *et al.*, 1987).

Reverse transcriptase inhibitors exhibit potent anti-HIV-1 activity. HIV inhibitors targeted at the virus-associated reverse transcriptase (RT) can be divided into two groups, depending on whether they are targeted at the non-substrate or substrate binding site. The first group of compounds, which do not require metabolic conversion, include certain benzodiazepinones (i.e. tetrahydro-imidazo[4,5,1,-jk][1,4]-benzodiazepin-2-(1H)-one derivatives, TIBO), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT),  $\alpha$ -anilinophenylacetamide derivatives, pyridinones (i.e., 3-[(arylmethyl)amino]pyridin-2(1H)-one), bis(heteroaryl)piperazines (BHAP), acyclouridines, and dipyridodiazepinones which interact noncompetitively with a specific allosteric binding site of HIV-1 RT (De Clercq *et al.*, 1992; Saunders J., 1992, De Clercq, 1992, Sandström *et al.*, 1993a, b). Non-nucleoside RT inhibitors that target the non-substrate binding site of the RT enzyme, induce rapid development of resistance which appears to be temporally related to loss of antiviral activity (Havir *et al.*, 1995).

2',3'-Dideoxynucleosides (ddNs) belong to the second group, that need to be phosphorylated intracellularly to their active triphosphate form before they act as competitive inhibitors or alternate substrates (chain terminators) of HIV RT. They interact at the substrate binding site of the enzyme where the deoxynucleoside triphosphates



(dNTPs) normally bind. There are currently five reverse transcriptase inhibitors approved for use in HIV disease (Figure 1.5). 2',3'-Dideoxynucleosides such as 2',3'-dideoxy-3'-thiacytidine (3TC, 4, lamivudine) (Soudeyns *et al.*, 1991), 2',3'-dideoxycytidine (ddC, 5, zalcitabine, Hivid) (Yarchoan *et al.*, 1988, Mitsuya 1986), 3'-azido-3'-deoxythymidine (AZT, 6, zidovudine, Retrovir) (Mitsuya *et al.*, 1985), 2',3'-didehydro-3'-deoxythymidine (d4T, 9, stavudine, Zerit) (Balzarini *et al.*, 1987b) and 2',3'-dideoxyinosine (ddI, 10, didanosine, Videx) (Yarchoan *et al.*, 1989) are used clinically to treat patients with human immunodeficiency virus (HIV) infection (Figure 1.5). 3'-Fluoro-3'-dideoxythymidine (FLT, 7), alovedine) is also an active reverse transcriptase inhibitor.

#### 1.4.1.1. Mechanism of action

These ddNs (structures shown in Figure 1.5) are prodrugs that must enter the infected cell and then be phosphorylated to the active triphosphate moiety by host cell kinases (Furman *et al.*, 1991) (Figure 1.7), like the natural deoxynucleosides whose functions they mimic. AZT (6), FLT (7) and d4T (9) serve as analogs of deoxythymidine, but ddC (5), 3TC (4) are analogs of deoxycytidine and ddI (10) is an analog of deoxyadenosine. As triphosphates, the ddNs act through inhibition of viral reverse transcriptase by means of substrate competition with natural deoxynucleosides and through chain termination of the nascent DNA being transcribed by the viral reverse transcriptase by means of incorporation of the dideoxynucleoside triphosphate (ddNTP) that lacks the 3'-hydroxyl group. The efficiency of nucleoside analog inhibition of HIV-1 replication is dependent on four major factors: (1) the efficiency of cellular uptake of the analog, (2) the efficiency of phosphorylation of the analog by cellular kinases, (3) the level of competitive inhibition enacted, and (4) the efficiency of analog binding to RT and subsequent incorporation into elongating viral DNA (Arts *et al.*, 1996). The 5'-triphosphate of nucleoside analogs may inhibit HIV-1 reverse transcription through two mechanisms: (1) competitive inhibition with the native dNTP for RT and (2) competitive inhibition of reverse transcriptase and chain termination of viral DNA due to the absence of a 3'-hydroxyl group. Both mechanisms are thought to be required for inhibition of HIV in host cells (Schinazi *et al.*, 1992b, Arts *et al.*, 1996) (Figures 1.7 and 1.8).



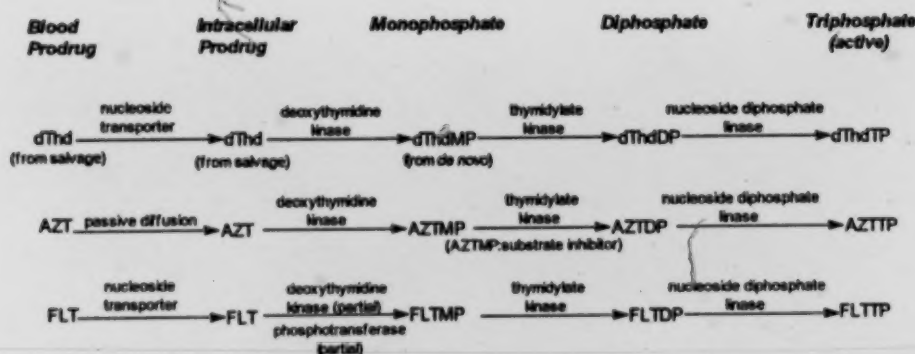


**Figure 1.7.** Cellular uptake and phosphorylation of antiretroviral nucleoside analogs. The major pathways of cellular uptake and anabolism (phosphorylation) of deoxythymidine (dThd, 8), zidovudine (AZT, 6) and allovudine (FLT, 7) as shown.

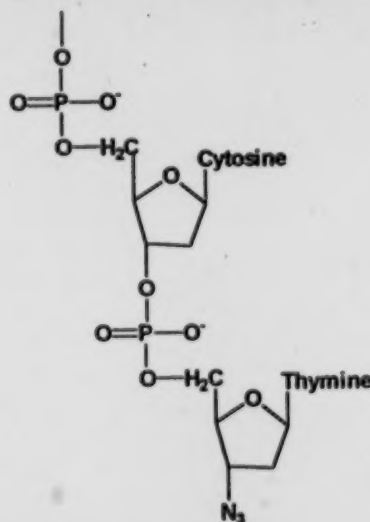


**Figure 1.8.** Terminated DNA chain. The azido group at the 3'-end position of the DNA chain prevents incorporation of a new nucleotide and results in DNA chain termination.

#### 1.4.1.2. Limitations in using 2',3'-dideoxynucleosides (ddNs)

The major problems with these drugs are their clinical toxicities such as bone marrow suppression and neuropathy, a low therapeutic index caused in part by inhibition of cellular polymerases, absolute dependence on host cell kinase-mediated activation (Figure 1.7)

which leads to poor activity, limited brain uptake, short half-life time in blood (the plasma half-life of AZT is approximately 1 h, and therefore frequent administration of AZT is necessary to maintain therapeutic drug levels), low potential for metabolic activation and/or high susceptibility to catabolism, and the rapid development of resistance to the drugs by HIV-1. Some important limitations are discussed briefly.

**1. Toxicity:** All licensed nucleoside analogs possess significant drug-specific dose-limiting toxicities, yielding a small therapeutic window between the minimally effective dose and the maximally tolerated dose. The more serious of these adverse effects include bone marrow toxicity (e.g., AZT, FLT), peripheral neuropathy (e.g., ddC, ddI, d4T), pancreatitis (e.g., ddI), myopathy (eg, AZT), and hepatic abnormalities (eg, AZT, ddI) (Martin *et al.*, 1994). The hematologic toxicities of AZT (6) and FLT (7) are reversible, dose or concentration dependent and occur within 1 month of starting either drug, and for AZT (6) at least, the toxicity worsens with advance stages of HIV disease. This cytotoxicity can be reversed by addition of other nucleosides without a change in AZT's antiviral effect (Bhalla *et al.*, 1989). Cellular DNA polymerase  $\alpha$  is 100-fold less resistant to zidovudine triphosphate but mitochondrial DNA polymerase  $\gamma$  is affected. 3'-Amino-3'-deoxythymidine (AMT), an amino metabolite of AZT (6), is toxic to granulocyte-macrophage and erythroid progenitor cells (Faraj *et al.*, 1994). One FLT (7) trial was terminated prematurely because of two unexplained cases of progressive hepatic failure (Flexner *et al.*, 1994). Further development of FLT (7) has been discontinued, suggesting that significant antiviral activity was not observed at plasma concentrations that were well tolerated in a controlled phase II study.

**2. Resistance:.** Resistant viruses can be detected soon after treatment. Multiple reverse transcriptase mutations have been shown to confer resistance to AZT (6), including mutations at amino acids 41, 67, 70, 215, and 219. Treatment with different nucleoside analogs may result in different mutations conferring resistance (Kellam *et al.*, 1995). It has become clear that HIV-1 burdens are substantial and high level virus replication occurs at all stages of infection. Even during asymptomatic and clinically quiescent periods, replication in lymphoid organs is extensive. The high virus turnover drives the pathogenic process and the development of genetic variation. Under the

selection pressure of drug therapy, viruses with resistance mutations accumulate, sometimes with complete replacement of wild-type virus by drug resistant mutants in plasma after only 2 to 4 weeks of therapy (Ho *et al.*, 1995, Wei *et al.*, 1995, Coffin *et al.*, 1995).

#### 1.4.1.3. Combination therapy

Combination therapy for controlling HIV-1 infection provides several potential advantages over monotherapy. Two or more drugs may have additive or synergistic interactions that produce better efficacy than either drug alone. Additive or synergistic combinations may also allow the use of drugs at lower doses than employed in monotherapy, possibly decreasing toxicity. Starting therapy with a combination regimen may also delay the emergence of resistant virus that can completely escape drug inhibition. If a virus resistant to one component is already present, broader coverage with combination therapy would also be useful. Combination therapy could target different cellular and tissue reservoirs of virus. Ongoing clinical trials are testing combinations, with the hope that different inhibitors may prevent or delay the development of resistance. Lamivudine (2'-deoxy-3'-thiacytidine, 3TC, 4) is a dideoxy analog of cytidine that is a relatively new RT inhibitor with potent antiretroviral activity *in vitro* with good oral bioavailability. 3TC (4) consists of a racemic mixture of  $\beta$ -D-(+)- and  $\beta$ -L-(-) enantiomers that have equipotent anti-HIV activity at nanomolar concentrations. 3TC was reported to be less toxic than AZT (6) and it is not cross-resistant to AZT (6) (Staszewski *et al.*, 1996a, b, Larder *et al.*, 1995). Resistance against 3TC (4) *in vitro* and *in vivo*, caused by a unique single base change at position 184, appears to fortuitously restore sensitivity to AZT (6) *in vitro*. HIV-1 isolates with this mutation show less growth *in vitro* than the wild type (Staszewski, 1996a, b, Larder *et al.*, 1995). Several recent clinical trials have shown remarkable sustained reductions of plasma viral RNA that is often below the limits of assay detection for prolonged periods. Different studies have generally employed three drug regimens that include two nucleoside reverse transcriptase inhibitors plus a third drug that is either a potent protease inhibitor or a nonnucleoside reverse transcriptase inhibitor. Examples include (indinavir, zidovudine, and lamivudine), (ritonavir, zidovudine, and

lamivudine) and (zidovudine, didanosine and nevirapine) (D'Aquila *et al.*, 1996, Staszewski *et al.*, 1996a, b, Carpenter *et al.*, 1996). Most combination therapies for HIV include AZT (6).

#### 1.4.1.4. 3'-Azido-3'-deoxythymidine (AZT, 6)

3'-Azido-3'-deoxythymidine (AZT, 6, BW A509U, Retrovir, zidovudine) was the first for clinical use in the treatment of infection with the human immunodeficiency virus (HIV) (Fischl *et al.*, 1987). AZT is a synthetic pyrimidine analog that differs from thymidine in having an azido substituent instead of a hydroxyl group at the 3' position of the deoxyribose ring. It was first synthesized by Horwitz *et al.* (1964) and later, by a modification of this procedure, by Lin and Prusoff (1978) as an anticancer agent. New methods have since been developed for the synthesis of AZT (6) (Fleet *et al.*, 1988, Chu *et al.*, 1988, Rao *et al.*, 1989, Czernecki *et al.*, 1991, Liotta *et al.*, 1995a, b, Huryn *et al.*, 1992, Balagopala *et al.*, 1996). The antiviral activity of AZT (6) was first reported by Ostertag *et al.* (1974). They found that AZT (6) inhibits the reverse transcriptase (RT) of Friend leukemia virus, whereas it had only a minimal effect on the replication of the host cell. Mitsuya *et al.* (1985) reported the inhibitory effect of AZT (6) on the infectivity and cytopathic effect of HIV *in vitro*.

AZT (6) prolongs survival and reduces morbidity. AZT (6) delays HIV progression and improves survival in HIV-1 infected patients with advanced disease. However, the beneficial effect of AZT (6) appears to diminish over time. Transmission of HIV-1 from mother to newborn can be reduced by AZT (6) (Connor *et al.*, 1994). Although AZT (6) effectively inhibits the replication of HIV-1 initiated by cell-free virus, it does not inhibit virus production by chronically infected cells or transmission of the infection to uninfected cells by syncytium formation (Manouilov *et al.*, 1995a, b).

##### 1.4.1.4.1. Mechanism of action and metabolism

On entering the cell, AZT (6) is phosphorylated to AZT monophosphate by the same cellular kinase which phosphorylates thymidine. AZT monophosphate is then phosphorylated to the diphosphate and triphosphate forms by other cellular kinases (Figure



1.7). AZT (6) is an efficient substrate for the cellular thymidine kinase, which converts it to AZT-MP in infected and uninfected cells. AZT-MP accumulates in cells because of its slow phosphorylation to AZT-DP by host cell thymidylate kinase, the rate-limiting step in AZT-TP formation. Based on *in vitro* studies, increasing extracellular concentrations of AZT (6), (i.e., picomolar to micromolar range) results in proportionally increasing AZT-MP concentrations but relatively small or no increases in AZT-TP. AZT-MP is a competitive inhibitor of thymidylate kinase and reduces the conversion of TMP to TDP, leading to decreased formation of TTP. The rate of AZT-MP phosphorylation to AZT-TP, catalyzed by thymidylate kinase, was only 0.3% that of TMP. AZT triphosphate is said to interfere with retroviral RNA dependent DNA polymerase (reverse transcriptase) thereby inhibiting viral replication. The growing DNA chains which incorporate AZT triphosphate are thereby prematurely terminated because AZT (6) cannot accept an additional nucleotide at the 3' position (Figure 1.8) (Balzarini, *et al.*, 1988, De Clercq *et al.*, 1994). Some studies have suggested that high levels of AZT-MP may impair HIV-1 RT RNase H activity (Tan *et al.*, 1991) and the 3'-exonuclease (Harrington *et al.*, 1993) that would otherwise cleave AZT-MP from the DNA 3'-terminal ends. Furthermore, AZT-MP inhibits protein glycosylation which may explain, at least in part, the cytotoxicity of AZT (6) (Hall *et al.*, 1994). Addition of thymidine reverses both the efficacy and toxicity of AZT (6), indicating the competition between thymidine triphosphate and AZT-TP for viral and host DNA polymerases. The normal metabolites of AZT (6) can inhibit HIV-1 integrase at concentrations achievable *in vitro*, suggesting the possibility that inhibition of viral integration may contribute to the *in vivo* potency of AZT (6) (Mazumder *et al.*, 1994).

Azidothymidine 5'-triphosphate (AZT-TP) is considered to be the metabolite responsible for the anti-HIV effect of AZT (6), via inhibition of reverse transcriptase, whereas cytotoxicity of AZT (6) correlates with AZT-MP levels (Törnevik *et al.*, 1995).

AZT (6) is also subject to catabolism. The bioavailability of AZT (6) after oral administration is about 63%. Metabolism studies indicated that three metabolites, 3'-amino-3'-deoxythymidine (11, AMT), 5'-*O*-glucuronide-3'-amino-3'-deoxythymidine (12, GAMT) and 5'-*O*-glucuronide-3'-azido-3'-deoxythymidine (13, GAZT) are formed, with



GAZT as the predominant metabolite (structures shown in Figure 1.9).

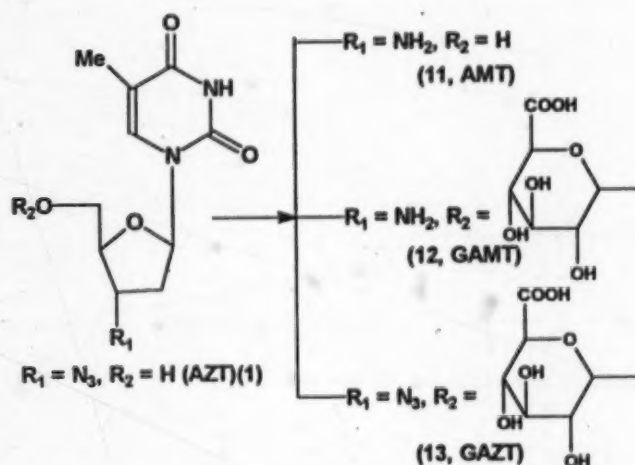


Figure 1.9. The chemical structures of AZT (6) metabolites.

#### 1.4.1.4.2. Pharmacokinetics

The pharmacokinetic disposition of AZT (6) in HIV-infected patients has been described in the literature (Blum *et al.*, 1988, Collins *et al.*, 1989, Klecker *et al.*, 1987, Moore *et al.*, 1995). AZT (6) was rapidly absorbed; the time to reach peak concentrations in plasma ranged from 0.6 to 0.8 h. The average oral bioavailability is 65%, with 17 to 23% of an intravenous (iv) dose excreted unchanged in urine (Morse *et al.* 1993, Klecker *et al.* 1987). The volume of distribution of AZT in humans at steady state is approximately 1.40 liter/kg, and its elimination half-life is approximately 1 h. AZT (6) is eliminated primarily by hepatic conjugation to an inactive glucuronyl derivative, GAZT. Approximately 60% of an iv dose is recovered as GAZT in urine. AZT (6) is not extensively protein bound (< 25%), and the clearance of AZT (6) in humans is approximately 1.63 Liter/h/kg of body weight, a value similar to hepatic blood flow (Moore *et al.*, 1995). While AZT (6) undergoes extensive glucuronidation in humans and monkeys to the 5'-O-metabolite (GAZT), it is principally excreted unchanged in lower species including dogs, rabbits, rats, and mice (Good *et al.*, 1986).

#### 1.4.1.5. 3'-Fluoro-3'-deoxythymidine (FLT, 7)

Langen *et al.* (1969) first synthesized alovudine (FLT, 7) and described its effects on carcinoma cells. It was also synthesized by other groups (Etzold *et al.* 1971, Huang *et al.*, 1991, Herdewijn *et al.*, 1987, 1989, Fleet *et al.*, 1988, Matthes *et al.* 1988, Green *et al.*, 1991, Huryh *et al.*, 1992; Liotta *et al.*, 1995a, b). Within the 3'-fluoro-2',3'-dideoxynucleosides, FLT (7) has proven to be the most potent anti-HIV agent (Balzarini *et al.*, 1988, Hartmann *et al.*, 1988, Matthes *et al.*, 1989, Herdewijn *et al.*, 1987, Bazin *et al.*, 1989). In general, FLT (7) is approximately 10 times more potent than AZT (6). The higher antiviral activity of FLT (7) is presumably due to its higher rate of intracellular phosphorylation to the triphosphate than AZT (6). FLT has a large therapeutic index, but it is toxic at 1-100  $\mu$ M concentration depending on the cell culture system used (Nasr *et al.*, 1992, Bergstrom *et al.*, 1992, Schinazi *et al.*, 1992b).

AZT-resistant viruses are not cross resistant to FLT (7). Alovudine (7) is active in cell cultures against zidovudine-resistant HIV. Development of resistance toward alovudine in cell cultures seems to be a very slow process (Sandström *et al.*, 1993a, b). The finding that FLT (7) is not cross-resistant to AZT (6) against AZT-resistant viruses, suggests that the mechanism of action of FLT (7) is not identical to AZT (6). Another possibility is that the 3'-fluoro moiety on FLT (7), which is smaller than 3'-azido group on AZT (6), can bind more effectively to AZT-resistant HIV-1 RT than AZT (6). The absence of cross resistance between FLT (7) and AZT (6) not only makes FLT (7) a potential alternative to AZT (6) as a single agent for the treatment of HIV infection, but it also increases the potential utility of FLT (7) in combination therapy. A combination of FLT (7) and AZT (6) shows a synergistic effect against HIV in H9 cell culture (Schinazi *et al.*, 1992b, Harmenberg *et al.*, 1990).

FLT 5'-triphosphate is also a potent inhibitor of endogenous hepatitis B virus (HBV) DNA polymerase and the production of HBV in HepG2 cells transfected with HBV DNA *in vitro* (Matthes *et al.*, 1990). The high degree of similarity between the reverse transcriptase of HIV and HBV makes it probable that combinations of FLT (7) and AZT (6) may also have synergistic effects against HBV (Pan *et al.*, 1992, Harmenberg *et al.*,

1990, 1996).

#### 1.4.1.5.1. Mechanism of action

The mechanism of action of FLT involves phosphorylation by cellular kinases to alovudine triphosphate (Matthes *et al.*, 1988) which inhibits HIV reverse transcriptase (Matthes *et al.*, 1985, Cheng *et al.*, 1987). No accumulation of monophosphate like that seen with zidovudine occurs. The rate of monophosphorylation of FLT (FLT-MP) was at least 200-fold less than that of AZT-MP. However, the rate of di- and triphosphorylation of FLT-MP was significantly augmented compared with that of AZT-MP (Matthes *et al.*, 1988, Cox *et al.*, 1992). Alovudine triphosphate seems to act both as a chain terminator of cellular DNA polymerase activity and as a competitive inhibitor to thymidine 5'-triphosphate (Matthes *et al.*, 1985) (Figure 1.7).

Cellular permeation of FLT (7) and dThd (8) are greater than of AZT (6). FLT (7) and dThd (8) permeated cell membranes by both a carrier-mediated mechanism (nucleoside transport system) and simple diffusion. This transport difference across the cell membrane between FLT (7) and AZT (6) may largely result from the fact that AZT (6) is more lipophilic than FLT (7) which may contribute to differences in extracellular and intracellular pharmacokinetic behavior (Kong *et al.*, 1992) (Figure 1.7).

#### 1.4.1.5.2. Pharmacokinetics

The ratio between alovudine (7) concentration in CSF and plasma was 0.15 at one hour after dosing in rhesus monkeys that was independent of the route of administration, suggesting that a nucleoside carrier-mediated process is involved in alovudine transport to the central nervous system. Absorption of FLT after oral administration was variable, with bioavailability ranging from 21 to 95% (Schinazi *et al.*, 1990).

A pharmacokinetic study of FLT (7) in plasma of monkeys after intravenous (iv) administration showed that the FLT (7) concentration in plasma declined over time with a half-life of 1.2 h. The steady-state volume of distribution was 0.93 liter/h/kg of body weight and the total clearance was 0.56 liter/kg. FLT (7) is excreted in urine predominately as unchanged drug (Kong *et al.*, 1992, Schinazi *et al.*, 1990, Boudinot *et*

*al.*, 1991, Bazin *et al.*, 1989, Hartmann *et al.*, 1988, Matthes *et al.*, 1987).

#### 1.4.1.5.3. Toxicity

Clinical trials using FLT suggested that thrombocytopenia and anemia are the main limiting toxic effects, resulting in a narrow therapeutic window. FLT (7) showed low cytotoxicity to MT4 cells with selectivity indices (efficacy/toxicity ratio) of  $> 47,000$  (Kong *et al.*, 1992). Cellular toxicity varies considerably, depending on the assay used, and is similar to that of zidovudine (Matthes *et al.*, 1988, Balzarini *et al.*, 1988).

High concentrations of FLT (7) result in a decreased dTTP pool size (Kong *et al.*, 1992, Cox *et al.*, 1990). Reduction in the endogenous pool size of dTTP by exposure of cells to high concentrations of FLT (7) may play a role in the cytotoxicity of FLT (7). A reduction in the level of dTTP, a competitive substrate to FLT-TP, for HIV-1 RT would facilitate binding of FLT-TP to HIV-1 RT and the host cellular DNA polymerases. In contrast with its high affinity for HIV RT, FLT-TP has poor affinity for DNA polymerase  $\alpha$  and moderate affinity for DNA polymerase  $\beta$  (Mansuri *et al.*, 1990, Kong *et al.*, 1992). Although FLT (7) bears a close structural relationship to 3'-azido-3'-deoxythymidine (AZT, 6), the 3'-substituent may be a determinant of clinical toxicity (Richman, 1987, Mamaeva *et al.*, 1994).

Persistent hematologic toxicity was observed in HIV-infected patients who were treated with FLT (7) (Ganes *et al.*, 1992), and as a result clinical trials were terminated. Sundseth and colleagues (1996) suggested that FLT (7) induces DNA fragmentation and apoptosis in a human cell line of hematopoietic origin, a possible explanation for the severe toxicity of FLT (7) observed *in vivo*. Fragmentation of DNA in CEM cells occurs at concentrations as low as 10  $\mu\text{M}$ .

#### 1.4.2. Fatty acids as antiviral and antifungal agents

Fatty acids exhibit their antiviral and antifungal activity by different mechanisms. In this thesis only the most important and probable mechanisms are discussed in detail. However, it is proposed that they act by a combination of these mechanisms. Fatty acids, in general, have been reported to inhibit cellular respiration (Rolinson *et al.*, 1954, Vicher



*et al.*, 1959, Pederson *et al.*, 1970), carbohydrate metabolism (Heseltine *et al.*, 1952), phosphate uptake (Samson *et al.*, 1955) and phospholipid metabolism (Vicher *et al.*, 1968). Finnerty (1989) concluded in his review that the uptake of fatty acids at a low concentration occurs by facilitated diffusion, while at high concentrations it undergoes uptake by simple diffusion. The differential activity of short and long chain fatty acids may be due to variations in solubility, incorporation into membranes, and in the degree and type of oxidation at different concentrations (Garg *et al.*, 1993).

The partial incorporation of fatty acids through membrane lipid biosynthesis may account for their differential activity (Garg *et al.*, 1993). Recently it has been proposed that inhibition of post-translational processing of viral and fungal precursor protein myristoylation, and more specifically myristoyl-CoA:protein *N*-myristoyltransferase (NMT), may be a more probable mechanism for the antiviral and antifungal activity exhibited by myristic acid analogs. It is necessary to emphasize again that NMT inhibition by myristic acid analogs may constitute only one of the several mechanisms responsible for the antiviral and antifungal activity of these analogs. However, there is substantial evidence supporting the NMT-inhibition mechanism. For example, studies with oxatetradecanoic acid analogs have already indicated that they enter mammalian, viral and fungal cells, are converted to their CoA thioesters by cellular acylCoA synthetases, and are delivered to target proteins by NMT. These targets include the Gag polyprotein precursor and Nef proteins of human immunodeficiency virus I and the Arf proteins of *C. albicans* and *Cryptococcus neoformans* (Schlesinger, 1993, Langner *et al.*, 1992).

#### 1.4.2.1. $\beta$ -Oxidation of fatty acids

In general, fatty acids are metabolized by  $\alpha$ -,  $\beta$ -, and  $\omega$ -oxidation by microbes (Klein *et al.*, 1971). Before entry into the citric acid cycle, acyl-CoA is oxidized via a  $\beta$ -oxidation pathway (Figure 1.10). This sequence of reactions is catalyzed by four enzymes, starting at the  $\beta$ -carbon atom of the fatty acid. Fatty acids are degraded by the sequential removal of two carbon units at a time, resulting in various 2-carbon fragments (acetyl-CoA). In the case of fatty acids with an even number of carbon atoms, the last degrading step results in acetyl-CoA formation, whereas for odd-numbered fatty acids, a 3-carbon fragment



(propionyl-CoA) is obtained as the final product (Van der wall, 1981).

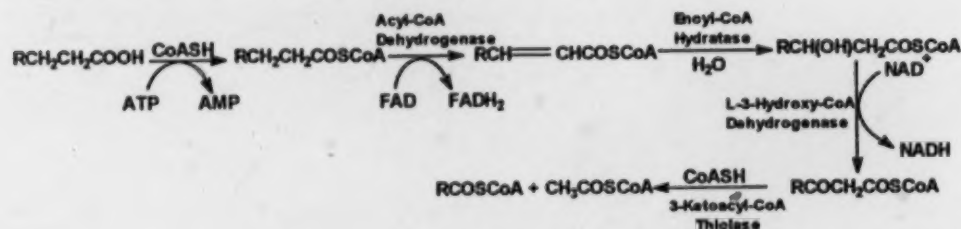


Figure 1.10.  $\beta$ -Oxidation of saturated fatty acids.

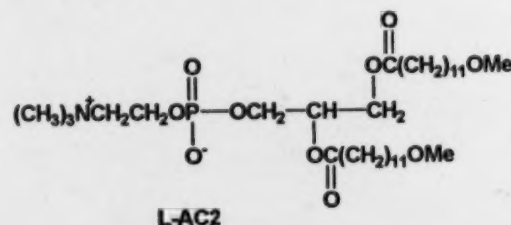
#### 1.4.2.1.1. Preventing fatty acid oxidation

Many fatty acids exhibit significant antifungal activity. In spite of their low toxicity, their systemic use has been ineffective, possibly because they are metabolized readily by the host via the usual fatty acid pathways. Pharmacokinetic studies indicate that 12-methoxydodecanoic acid (**66e**) is well absorbed from the gastrointestinal tract and that the clearance rate from plasma is rapid. The major route of metabolism for **66e** is via  $\beta$ -oxidation (Bryant *et al.*, 1993).

These observations present a challenge to the medicinal chemist since derivatives of "lead compounds" must retain their ability to (1) efficiently cross cell membranes, (2) serve as substrates for cellular acylCoA synthetase and myristoylCoA:protein *N*-myristoyltransferase, (3) be selectively incorporated into target proteins of interest, and (4) change the biological properties of the target protein. Moreover, it is difficult to correlate metabolic processing of a compound which occurs rapidly with potential therapeutic processing and therapeutic efficacy (Bryant *et al.*, 1993).

One way to slow fatty acid oxidation may be the esterification of fatty acids using a suitable alcohol. 12-Methoxydodecanoic acid (**66e**) appears to produce a concentration-dependent decrease in reverse transcriptase activity and syncytia formation (Bryant *et al.*, 1989). The diacylated *L*- $\alpha$ -phosphatidylcholine analog containing 12-methoxydodecanoic acid in both alkyl chains (*L*-AC2) may be used to slow **66e** oxidation. *L*-AC2 (i) has a 15-fold higher antiviral selectivity, (ii) is 7-fold more potent, and (iii) is 10-100-fold more synergistic with azidothymidine than 12-methoxydodecanoic acid (**66e**) alone. Because of

potent synergism, the antiviral selectivity of the diacylated L- $\alpha$ -phosphatidylcholine analog is  $> 10^4$  when coadministered with azidothymidine.



The increased antiviral activity and the increased synergism of a combination of L-AC2 with AZT, compared to 66e and AZT, is undoubtedly due to the cellular disposition of phospholipid analogs (Pidgeon *et al.*, 1993).

#### 1.4.2.2. Antifungal properties of fatty acids (FAs)

Several investigators have reviewed the antifungal activity exhibited by fatty acids (Table 1.1).

Major factors that may influence the fungitoxicity of fatty acids and their derivatives are the partition coefficient, the absence or presence of adsorbents such as albumin in the growth medium, chain length, *pKa* of the acid and pH of the medium (Table 1.2).

#### 1.4.2.3. Fatty acids as inhibitors of myristoylCoA:protein *N*-myristoyltransferase (NMT): a new perspective for antifungal and antiviral therapy

##### 1.4.2.3.1. Lipid Modification of Proteins

The covalent attachment of long chain fatty acids to proteins has been known for more than two decades. The first *N*-myristoylproteins were described in 1982 (Carr *et al.*, 1982, Aitken *et al.*, 1982). Covalent addition of the relatively rare C14 fatty acid, myristic acid, has been detected in a large number of viral and cellular proteins. The pathways for covalent addition of these lipids to the protein are relatively clear at this time. In fact, many of these biochemical activities can be effectively blocked by a variety of inhibitors which can function both *in vitro* and *in vivo* (Schlesinger *et al.*, 1993).

Table 1.1. Antifungal properties of fatty acids.

| REFERENCE                                                   | Fatty Acids                                             | Antifungal Properties                                                                                                                                                                            |
|-------------------------------------------------------------|---------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Clark <i>et al.</i> , 1899                                  | FA(s) in general                                        | First to report the antifungal properties of FA(s) (Effect of acetic acid against <i>A. niger</i> )                                                                                              |
| Kiesel <i>et al.</i> , 1913,<br>Garg <i>et al.</i> , 1993   | Saturated FA(s)                                         | Antifungal activity and chain length relationship (C 1:0 to C 11:0 series)                                                                                                                       |
| Peck <i>et al.</i> , 1938,<br>Thornton <i>et al.</i> , 1963 | Saturated FA(s)                                         | No relationship between antifungal activity and chain length (C 1:0 to C 10:0 series)                                                                                                            |
| Barash <i>et al.</i> , 1967                                 | Stearic and palmitic acids                              | Inactive against <i>Microsporium gypseum</i> and other dermatophytes                                                                                                                             |
| Gershon <i>et al.</i> , 1977                                | Esters of<br>2-bromo-3-fluorosuccinic acid              | Relationship between length of the ester and fungitoxicity against <i>C. albicans</i> , <i>A. niger</i> , <i>Mucor mucedo</i> , and <i>T. mentagrophytes</i> $C_2 > C_1 > C_3 > C_4 > C_5 > C_6$ |
| Gershon <i>et al.</i> , 1976a                               | $\alpha,\omega$ -Alkanedicarboxylic acids               | Poor antifungal activity <i>A. niger</i> , <i>T. viridae</i> and <i>M. verrucaria</i>                                                                                                            |
| Gershon <i>et al.</i> , 1976b                               | 4-Substituted crotonic esters                           | Antifungal activity against <i>A. niger</i> and <i>T. mentagrophytes</i> $I > Br > Cl > CH_3S > CH_3O > F = H$                                                                                   |
| Gershon <i>et.</i> , 1978a                                  | Halofumaric Acids                                       | Antifungal activity against <i>C. albicans</i> and <i>A. niger</i>                                                                                                                               |
| Swenson <i>et al.</i> , 1980                                | 2-Fluorinated fatty acids<br>and nonfluorinated analogs | Antifungal activity against <i>A. niger</i> and <i>T. viridge</i> was dependent on concentration of the fatty acid and its chain length, but not $pK_a$ of the fatty acid.                       |
| Garg <i>et al.</i> , 1993                                   | Saturated fatty acids                                   | Short chain fatty acids (C7-C10) are more toxic to dermatophytes than long chain analogs (> 12).                                                                                                 |

Table 1.2. Structure-activity relationships of antifungal fatty acids.

| Reference                             | Factor                     | SARs                                                                                                                                                       |
|---------------------------------------|----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Gershon <i>et al.</i> , 1978a-c, 1973 | Weight basis, Chain length | 2-Alkynoic acids > 2-alkenoic acids > alkanolic acids without halogen substitution > 2-bromoalkanoic acids > 2-fluoroalkanoic acids > n-alkoxyacetic acids |
| Gershon <i>et al.</i> , 1973          | pH                         | Decreasing the pH increases fungitoxic action of the carboxylic acids.                                                                                     |
| Gershon <i>et al.</i> , 1973          | pKa                        | The pKa of the acid does not affect fungitoxicity significantly.                                                                                           |
| Gershon <i>et al.</i> , 1976a-b, 1978 | Partition coefficient      | Fundamental determinant for fungitoxicity                                                                                                                  |
| Gershon <i>et al.</i> , 1978a-c       | Adsorbent such as albumin  | Deactivating effect of the adsorbent due to hydrogen bonding                                                                                               |

#### 1.4.2.3.1.1. N-Myristoylation of Proteins

Protein *N*-myristoylation (Figure 1.11) is an early event in acylprotein biosynthesis. Addition of inhibitors of protein synthesis to cells blocks protein *N*-myristoylation as rapidly as measurements can be made. This suggests that myristoylation occurs either cotranslationally or very soon after the completion of polypeptide synthesis (Towler *et al.*, 1988). Protein *N*-myristoylation in viruses and fungi is catalyzed by myristoyl-CoA:protein *N*-myristoyltransferase (NMT) (E.C. 2.3.1.97) that results in the co-translational linkage of myristic acid (C14:0), via an amide bond, to NH<sub>2</sub>-terminal glycine (Gly) residues of a variety of viral and fungal proteins following the removal of the initiator methionine residue by methionylpeptidase (Paige *et al.*, 1990) (Figure 1.11).

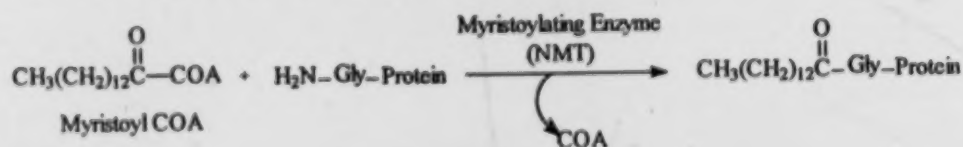


Figure 1.11. Protein myristoylation.

Myristoyl-CoA is synthesized via esterification of free myristic acid by CoA in a two-step reaction catalyzed by acyl-CoA synthetase (EC 6.2.1.3) (Figure 1.12) (Johnson *et al.*,

1994).

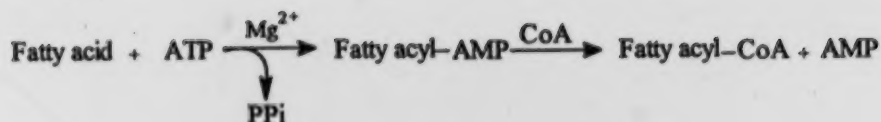


Figure 1.12. Synthesis of fatty acyl-CoA.

The reaction mechanism is highly ordered. A binary complex of NMT:myristoyl-CoA is formed initially that is followed by subsequent binding to a peptide substrate. Formation of a myristoyl-CoA.NMT.peptide ternary complex is then followed by transfer of myristate from CoA to the peptide (Rocque *et al.*, 1993) (Figure 1.13).

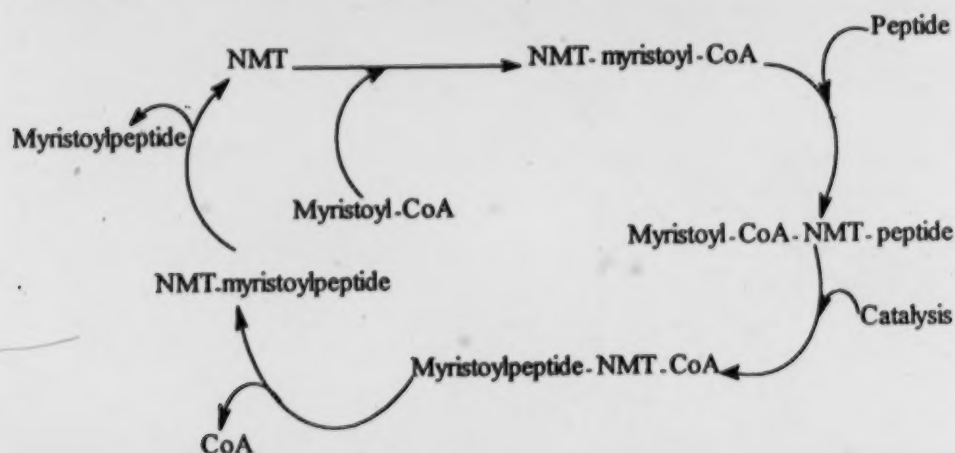


Figure 1.13. Myristoylation of peptides by myristoyl-CoA:protein *N*-myristoyltransferase (NMT).

In this sequential ordered mechanism, binding of the first substrate (myristoyl-CoA) greatly enhances the binding of the second substrate (peptide) (Rocque *et al.*, 1993). After catalytic transfer of myristate from CoA to the peptide, CoA is released followed by release of myristoylpeptide from NMT.

This mechanism predicts that regulation of cellular myristoyl-CoA pools and their availability to NMT should play a critical role in regulating protein *N*-myristoylation *in vivo* (Rocque *et al.*, 1993, Devadas *et al.*, 1992).



Myristoyl-CoA:protein *N*-myristoyltransferase (NMT, E.C. 2.3.1.97) is therefore an attractive target for antiviral and antifungal therapy. Since the peptide substrate specificity of fungal, viral and mammalian NMTs are different, it may be possible to design specific inhibitors for the fungal and viral enzyme (Rocque *et al.*, 1993, Duronio *et al.*, 1995).

#### 1.4.2.3.1.1.1. Myristoylated Fungal Proteins

Many fungal species like *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans* synthesize a variety of *N*-myristoyl proteins using NMT as catalyst. These fungal myristoylated proteins include ADP ribosylation factor (Arf1p, Arf2p) in *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans* (Langner *et al.*, 1992).

*Saccharomyces cerevisiae* produces at least 12 *N*-myristoylproteins (Duronio *et al.*, 1991) and requires a functional *N*-myristoyltransferase gene (NMT1) for viability (Towler *et al.*, 1987). At the present time, *S. cerevisiae* is an attractive organism for identifying potential therapeutic targets for antifungal therapy.

Genetic studies have revealed that myristoylCoA:protein *N*-myristoyltransferase is an essential enzyme for *Candida albicans*. *Candida albicans* contains at least two *Arf* genes. One of the *Arf* proteins is known to be a substrate for NMT. It may be possible to design species-specific inhibitors of *C. albicans* NMT which could function as fungistatic or fungicidal agents. Biochemical analyses have indicated that selective inhibitors could be developed by exploiting differences in peptide substrate specificities of human and fungal NMTs (Weinberg *et al.*, 1995). Similarly, *C. neoformans* and human NMTs exhibit differences in their peptide substrate specificities. These differences can be exploited to develop a new class of fungicidal drugs (Lodge *et al.*, 1994).

#### 1.4.2.3.1.1.2. Myristoylated viral proteins

Covalent modification of eukaryotic viral proteins with myristic acid has been observed in an increasing number of DNA and RNA virus families (Chow *et al.*, 1993). Addition of a tetradecanoyl group is essential for assembly/replication of some of these viruses or for production of infectious virions (Kishore *et al.*, 1993). Tetradecanoyl

attachment occurs at the amino terminal of a newly exposed glycine residue for a variety of structural and nonstructural proteins encoded by the genomes of enveloped viruses (retroviruses, hepadnaviruses, poxviruses and herpesviruses) and nonenveloped viruses (papovaviruses, picornaviruses and reoviruses) (Schlessinger *et al.*, 1993, Rocque *et al.*, 1993, Kishore *et al.*, 1993, Devadas *et al.*, 1993, Langner *et al.*, 1992, Ravanello *et al.*, 1994, Chow *et al.*, 1987, Moscufo *et al.*, 1992, Shoji *et al.*, 1988).

Covalent myristoylation of eukaryotic viral proteins by enveloped retroviruses and hepadnaviruses that are discussed here has been observed. From retroviruses e.g. Pr160<sup>gag-pol</sup>, Pr55<sup>gag</sup>, the capsid protein P17 and Nef proteins (Schultz *et al.*, 1983a, b, Tsai *et al.*, 1985, Veronese *et al.*, 1988, Henderson *et al.*, 1988, Schultz *et al.*, 1984, Schultz *et al.*, 1985, Buss *et al.*, 1985) in human immunodeficiency virus 1 (HIV-1) from hepadnaviruses e.g. PreS1 protein in the membrane envelope of hepatitis B (HBV) and surface (S) protein and presurface (S) protein in the envelope of duck hepatitis B virus (DHBV) (Macrae *et al.*, 1991, Towler *et al.*, 1988, Rocque *et al.*, 1993, Kuroki *et al.*, 1989, Lanford *et al.*, 1989, Persing *et al.*, 1987, Gavilanes *et al.*, 1982) are myristoylated. Myristoylation of retrovirus gag polypeptides is essential for either the assembly or budding of virus particles at membranes and for the release of preformed capsids. Myristoylation of the preS protein of duck hepatitis B virus is required for virion infectivity, but not for assembly or release (Marc *et al.*, 1990).

#### **1.4.2.3.1.1.2.1. Gag myristoylated Proteins in Human Immunodeficiency Virus (HIV)**

It is known that myristate (C<sub>14</sub>) becomes covalently bound to soluble and membrane proteins via an amide linkage early in the protein biosynthetic pathway of HIV. The HIV-1 genome encodes different myristoylated proteins such as precursor Pr55<sup>gag</sup>, p17<sup>gag</sup> and p27<sup>nef</sup> (Bryant *et al.*, 1993).

The Gag polypeptides of HIV-1 must be myristoylated for transport to the membrane bilayer and to produce normal virion assembly and infectious particle production. When acyl CoA's of the wrong chain length are bound, dramatic changes occur in NMT's affinity for peptide substrates. It has been demonstrated that replication of HIV-1 can be inhibited

by heteroatom-containing analogs of myristic acid without accompanying cellular toxicity (Devadas *et al.*, 1993, Rocque *et al.*, 1993).

#### **1.4.2.3.1.1.2.2. Protein myristoyl-modified non-structural viral proteins (negative factor, Nef)**

The HIV-1 genome encodes a 197-210 residue *N*-myristoylated, non-virion protein known as negative factor (Nef). Nef down-regulates CD4 by endocytosis and lysosomal degradation and shows to have kinase activity. CD4 downregulation is strongly enhanced by the association of Nef with cell membranes through myristoylation. Nef has also been shown to increase the infectivity of produced virions. The two functions of Nef may be related, in that down-regulation of CD4 may promote the production of HIV-1 that has greater infectivity (Schwartz *et al.*, 1995, Miller *et al.*, 1995). Nef appears to be located outside of the nucleus, in association with cell membranes and the cytoskeletal matrix (Bryant *et al.*, 1993).

Based on the above observations, several analogs of fatty acids have been synthesized and evaluated to examine their activity against HIV-1 *in vitro*.

#### **1.4.2.3.1.1.2.3. Myristoylated proteins in Hepatitis B Virus**

PreS1 protein is myristoylated such that its synthesis is inhibited by unnatural fatty acids. The membrane envelope of hepatitis B virus (HBV) particles contains three related viral proteins (L, M, and S) (Persing *et al.*, 1987). The L protein is preferentially localized within virions rather than in subviral particles, and is required for virion formation and release. Although all information necessary for particle assembly and export appears present in the S protein sequence (Kuroki *et al.*, 1989, Chow *et al.*, 1993, Bruss *et al.*, 1991, Prange *et al.*, 1991), HBV particle formation occurs in the presence or absence of the myristoyl moiety. Even though myristoylation-null HBV mutants form virion particles, these particles are non-infectious (Prange *et al.*, 1991). Similarly, myristoylation of the preS protein of duck hepatitis B virus is required for virion infectivity, but not for assembly or release (Marc *et al.*, 1990).

#### 1.4.2.3.1.2. Functional role of myristoylated proteins

The myristoyl moiety acts as a membrane targeting/association signal and the myristoyl moiety is important for the structural integrity of the modified protein (Persing *et al.*, 1986). *N*-terminal myristoylation has been shown to be essential for the development of *C. albicans*, *C. neoformans*, HIV and HBV. While the precise role of covalently bound myristate in these maturation processes is not known, it is thought in HIV to facilitate the proper Pr55<sup>gag</sup> subcellular targeting and Gag-membrane/protein interactions which precedes and/or facilitates core assembly and precursor Gag proteolytic processing during final virus maturation (Burnette *et al.*, 1992). On the other hand, within an infected cell, the myristic acid is added co-translationally. All other lipid modifications occur post-translationally after synthesis and initial folding of the protein is complete. Thus, unique from other lipid modifications, the myristoyl moiety is present during the folding of the nascent polypeptide chain and probably strongly participates in forming the overall secondary and tertiary structure of the synthesized protein. The myristoyl moiety is present for the life-time of the viral protein. Thus, the presence of the myristic acid must be structurally and functionally compatible with the potentially different functional role of the viral protein at all stages of the viral life cycle. In addition, mechanistically, the exact role or the relative functional importance of the myristoyl moiety during viral infection may vary from stage to stage. The myristoyl moiety is important in viral replication and appears to affect proper function of the structural proteins for each virus family (Chow *et al.*, 1993).

From this perspective, one could speculate that the presence of a myristoyl moiety could also effect the folding kinetics of proteolytic processing, and could affect the ease with which a virion particle (or protein complex) changes from one structural conformation to another (Chow *et al.*, 1993).

The antiviral and antifungal activities of several fatty acids and antiviral activity of their corresponding 5'-*O*-ester prodrugs will be discussed in the results section.

#### 1.5. Prodrugs

The term prodrug was first introduced by Albert in 1958 to describe compounds which



are pharmacologically inactive derivatives of an active agent, which undergoes chemical or enzymatic biotransformation resulting in the release of the active drug after administration. The metabolic product or parent drug subsequently elicits the desired pharmacological response (Albert *et al.*, 1958, Stella *et al.*, 1975, Silverman *et al.*, 1992). This allows the prodrug to bypass the barrier and, once past the barrier, to revert to the parent compound by postbarrier enzymatic or nonenzymatic processes (Figure 1.14).

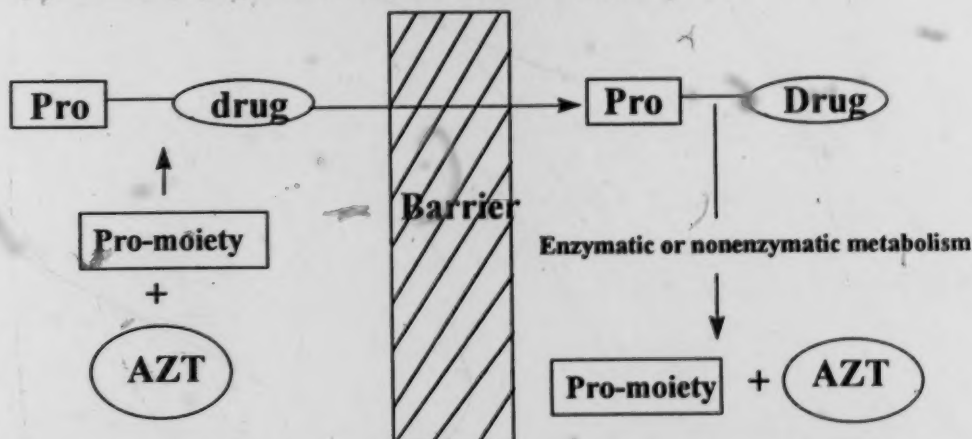


Figure 1.14. The principle of the prodrug concept as a means of improving the delivery of AZT.

A prodrug should not be confused with a soft drug, which is active, but is inactivated in a controlled fashion. Clearly, a prodrug cannot be identified by *in vitro* screening unless some activation mechanism is included.

There are numerous reasons why one may wish to utilize a prodrug strategy in drug design. Some of the applications are: to improve a specific deficient physicochemical property such as, water solubility, stability or membrane permeability, to improve pharmacokinetic problems (e.g., low absorption, distribution, retention time in plasma or low oral bioavailability), to improve drug delivery (e.g. specific-site targeting; drug localization), to regulate transport, to confer sustained release of a drug (prolonging or shortening the action), to decrease toxicity and poor patient acceptability, and to formulate the drug in proper form. One important membrane that often is targeted for drug delivery is the blood-brain barrier. Also, once the drug has entered the brain, it must



be modified so that it does not escape (Silverman *et al.*, 1992).

### 1.5.1. Different strategies for designing prodrugs

Carrier-linked prodrugs usually have a labile group, similar to a protecting group used in organic synthesis, which is cleaved to release the active drug. These can be sub-classified as into bipartate (one carrier and the drug), and tripartate, in which the carrier is connected via a linker to the drug (Silverman *et al.*, 1992).

There are different strategies for designing prodrugs that include esters (Stella *et al.*, 1975, Silverman *et al.*, 1992), amides (Pitman, 1981), structures based on a reductive or oxidative process (Bodor N., 1981a, b), decarboxylation (Wade *et al.*, 1975),  $\alpha$ -acyloxyalkyl derivatives, and *N*-Mannich bases (Bundgaard *et al.*, 1981). Alternative strategies are *N*-alkylation or *N*-hydroxymethylation of amides (Pitman *et al.*, 1981, Bundgaard *et al.*, 1981) and regeneration of a double bond via an elimination reaction (Kumar *et al.*, 1994). Most synthetic prodrugs are ester or amide derivatives. Several strategies, to be discussed later, have been employed to design prodrugs to improve the delivery of AZT (6) and selected ddNs to the brain or other organs (Bodor *et al.*, 1981a, b, Kawaguchi *et al.*, 1990, Aggarwal, *et al.*, 1990, Piantadosi, *et al.*, 1991, Hostetler *et al.*, 1990a, b, Steim *et al.*, 1990, Torrence *et al.*, 1988, Chu *et al.*, 1990, Palomino *et al.*, 1989, McGuigan *et al.*, 1990a, b).

### 1.5.2. Prodrugs of AZT

Most prodrug derivatives of AZT were prepared, by derivatization of AZT (6) at the 5'-O position. However, other approaches have also been utilized that encompass 5-halo-6-alkoxy-5,6-dihydro derivatives of AZT (6) and FLT (7) (Wiebe *et al.*, 1995, Kumar *et al.*, 1996).

#### 1.5.2.1. 5,6-Dihydro prodrugs of AZT

These 5,6-dihydro derivatives were found to be more lipophilic than the parent compound, and the 5-iodo and 5-bromo analogs of AZT and FLT exhibited activities comparable to AZT (6) and FLT (7), respectively. After regeneration of the 5,6-olefinic

bond, the more hydrophilic parent nucleoside will be locked inside the cell and exerts its pharmacological effect in a more sustained manner. 5-Halo-6-methoxy-5,6-dihydro prodrugs of AZT (14, 15) were designed in order to improve brain delivery of AZT (6) (Wang *et al.*, 1996).

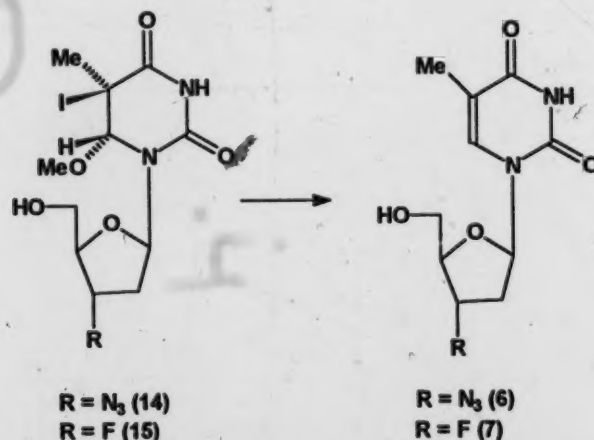


Figure 1.15. Regeneration of the 5,6-olefinic bond from 5-halo-6-methoxy-5,6-dihydro prodrugs.

#### 1.5.2.2. 5'-O-ester prodrugs

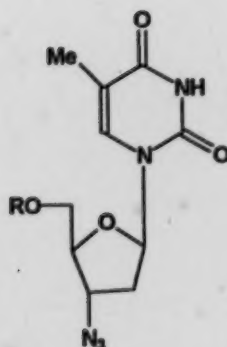
Esters have dominated prodrug research because they possess ideal characteristics for bio-derivatives. Esters exhibit reasonable chemical stability *in vitro*, which allows them to be prepared as formulations with adequate shelf lives. In addition, by virtue of their ability to function as enzyme substrates, they exhibit a suitable *in vivo* lability as well (Bundagard *et al.*, 1985, 1989).

Esterification of the 5'-hydroxyl group is a common approach to improve the brain uptake and *in vivo* efficacy of anti-HIV nucleoside derivatives. Several groups have used the ester prodrug approach at the 5'-position of the nucleosides (Aggarwal *et al.*, 1990, Torrence *et al.*, 1993, Kawaguchi *et al.*, 1990, Palomino *et al.*, 1989, Chu *et al.*, 1990). Different categories of 5'-O-esters of AZT (6) are discussed here. Several prodrugs of AZT (6) were synthesized by conjugating AZT (6) with various compounds such as (1) the carboxylic acid retinoic acid (Kawaguchi *et al.*, 1990, Seki *et al.*, 1990, Aggarwal *et*

*al.*, 1990) and amino acids (Aggarwal *et al.*, 1990, Heiati *et al.*, 1997), (2) phospho derivatives such as glycosyl phosphotriesters (Henin *et al.*, 1991, Namane *et al.*, 1992), lipids (Piantadosi *et al.*, 1991), and masked phosphate nucleotides (phosphodi- or triesters) (Namane *et al.*, 1992, McGuigan *et al.*, 1993b, Hostetler *et al.*, 1990a, b, Balagopala *et al.*, 1994) and (3) another anti-HIV moiety (Piantadosi *et al.*, 1991). In these prodrugs, the conjugating moiety is linked to AZT (6) via a 5'-*O*-ester or 5'-*O*-phosphate group. 5'-*O*-esters of AZT (6) have been designed to improve their pharmacokinetic profile and enhance their uptake in target tissues. In comparison to AZT (6), some prodrugs exhibited similar anti-HIV activity with much improved pharmacokinetic properties such as increased plasma half-life, lipophilicity and cell uptake, and decreased toxicity.

In general, at best, derivatives of AZT (6) have a similar *in vitro* antiviral potency to that of the parent nucleoside (McGuigan *et al.*, 1991b). The obvious drug-design rationale for this approach is that the conjugate will be converted by hydrolysis or enzyme action to AZT (6) itself or its 5'-monophosphate. Examples of derivatives that do indeed display *in vitro* anti-HIV activity include 5'-alkylamido ether phospholipid (Piantadosi *et al.*, 1991), the 5'-methylphosphonic acid (Tarusova *et al.*, 1990), the CF<sub>3</sub>CH<sub>2</sub> and CCl<sub>3</sub>CH<sub>2</sub> 5'-trialkyl phosphates (McGuigan *et al.*, 1990c), a 5'-dimyristoylphosphatidyl derivative (Hostetler *et al.*, 1991), the 5'-monophosphate (Tarusova *et al.*, 1989), a 5'-monophosphate diglyceride (phosphatidic acid conjugate) (Steim *et al.*, 1990), a sugar *n*-C<sub>16</sub>H<sub>33</sub> 5'-phosphate triester (Henin *et al.*, 1991), and the 5'-[(1,4-dihydro-1-methyl-3-pyridinyl)carbonyl]ester (Cogu *et al.*, 1989, Torrence *et al.*, 1988). Analog diester conjugates with ddi (10), or with another molecule of AZT (6) (Busso *et al.*, 1988), also showed activity.

In the following sections a general formula for 5'-*O*-ester derivatives of AZT will be used. The different substituents **R** at 5'-position will be introduced in each section.



#### 1.5.2.2.1. Carboxylic esters (14-22)

##### 1.5.2.2.1.1. Steroid esters (e.g. 16)

Sharma *et al.* (1993) synthesized three 5'-O-esters of AZT (6) with three different inactive steroidal 17 $\beta$ -carboxylic acids (see 16 in Figure 1.16). Since the steroidal acids chosen retain some of the glucocorticoid structural features such as 3-keto, 4-ene and 11-hydroxy groups, the conjugates were expected to bind to transcortins which are plasma glucocorticoid transport proteins. The conjugate-protein complex would be protected from metabolic transformation in plasma, thus prolonging the half-life of the nucleoside. All conjugates were active as anti-HIV agents and showed activity and toxicity comparable to those of AZT (6) (Balagopala, *et al.*, 1995).

##### 1.5.2.2.1.2. Adamantane conjugates (e.g. 17)

Prodrugs conjugated to a 1-adamantane moiety via an ester bond were synthesized to improve the transport of AZT (6) into the CNS by Tsuzuki *et al.* (1994) (see 17 in Figure 1.16). When these prodrugs were administered intravenously to rat, the prodrugs in brain tissue were detected at a 7-18 times higher concentration than AZT (6) even though there was a negligible amount of prodrug in the cerebrospinal fluid (Tsuzuki *et al.*, 1994).

##### 1.5.2.2.1.3. Chemical delivery system

##### (1,4-Dihydro-1-methyl-3-[(pyridylcarbonyl)oxy]ester) (18)

Increasing the lipophilicity of a compound to an optimal level, using the prodrug

approach, facilitates the delivery of a compound to its target site of action. However, this may not be a specific process since in general, all organs are often exposed to a greater burden. This is especially important with cytotoxic agents. While increased membrane permeability makes a compound more brain-selective, there is almost always a disproportionate rise in systemic toxicity. These two objections to simple antiviral prodrugs, viz 1) increased tissue burden with little tissue specificity, and 2) poor CNS retention, illustrate the need for a more sophisticated approach such as a chemical delivery system for brain-targeted delivery (Bodor N., 1992). A chemical delivery system (CDS) which was proposed initially by Bodor *et al.* in 1981, is a popular approach to deliver drugs to the brain. This approach involves the chemical transformation of the quaternary

R

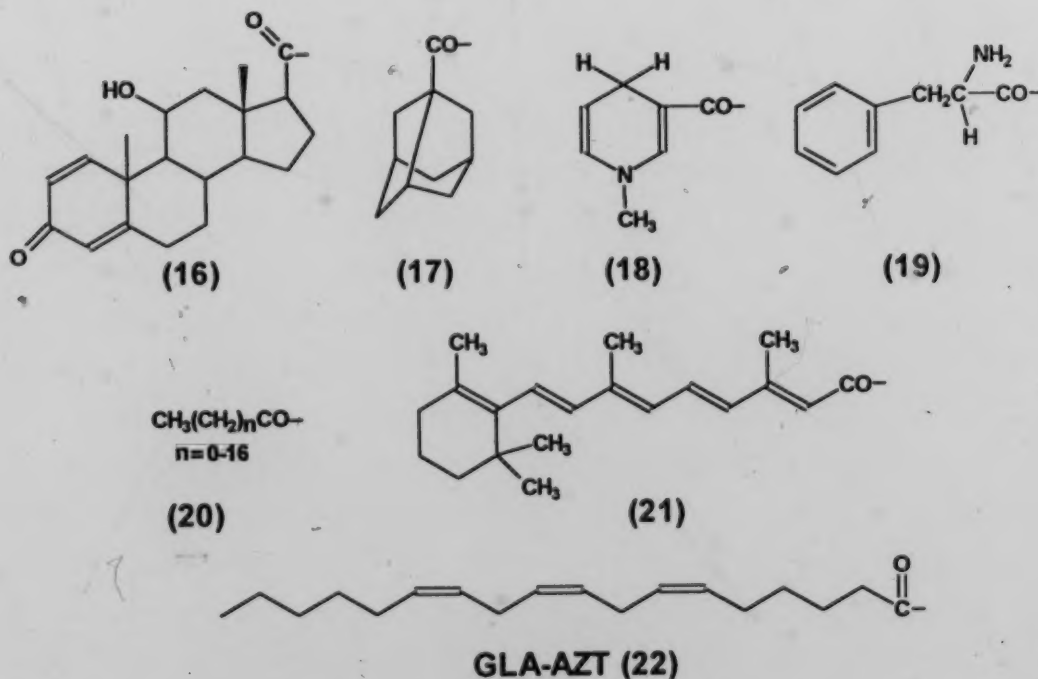


Figure 1.16. 5'-O-esters of AZT.

form of a drug  $[\text{Q}^+]$ , which normally does not penetrate the blood-brain barrier, to a



reduced lipid soluble form [HQ]. After intravenous administration, the lipid-soluble [HQ] is readily distributed throughout the body and readily crosses the intact blood-brain barrier. Subsequently, the  $\text{NAD} \rightarrow \text{NADH}$  redox system regenerates the original impermeable form [ $\text{Q}^+$ ] from [HQ] in the brain. Oxidation of the carrier moiety to the hydrophilic pyridinium salt (quaternized salt) in the brain slows its elimination due to a lock-in mechanism of polar compounds in the brain, while elimination from the general circulation of the hydrophilic pyridinium salt is accelerated. Subsequent cleavage of the quaternary carrier drug results in sustained retention of the drug in the brain with a reduction in general toxicity due to the rapid elimination of the polar quaternary salt (Figure 1.17). Chemical delivery systems (CDS's) based on a dihydropyridine-quaternary pyridinium ion redox system could be used for specific delivery of AZT (6) into the brain (see 18 in Figure 1.16) (Bodor *et al.*, 1981a, Kumar *et al.*, 1993, Morin *et al.*, 1993, Torrence *et al.*, 1988, Palomino *et al.*, 1989, Chu *et al.*, 1990, Gogu *et al.*, 1989, Brewster *et al.*, 1991, Aggarwal *et al.*, 1990, Agrawal, 1991, Little *et al.*, 1990, Lupia *et al.*, 1993, Pop *et al.*, 1995).

*In vivo* studies indicated that a significantly greater amount of AZT (6) could be delivered to the brain following administration of a prodrug than the parent drug. However, constant lower concentrations of AZT (6) (50% reduction) were present in plasma after prodrug administration compared to those after AZT (6) dosing (Palomino *et al.*, 1989). Unfortunately, the dihydropyridine-containing derivatives suffer from stability problems, since even in the dry state they are very sensitive to oxidation as well as to water addition. Such problems have significantly complicated attempts to commercialize the system. Thus, a different carrier approach to brain-targeted drug delivery which would not include the inherently unstable dihydropyridine system would be desirable (Bodor N., 1992). Oral administration of these analogs is also less suitable due to their instability at the low pH of the stomach.

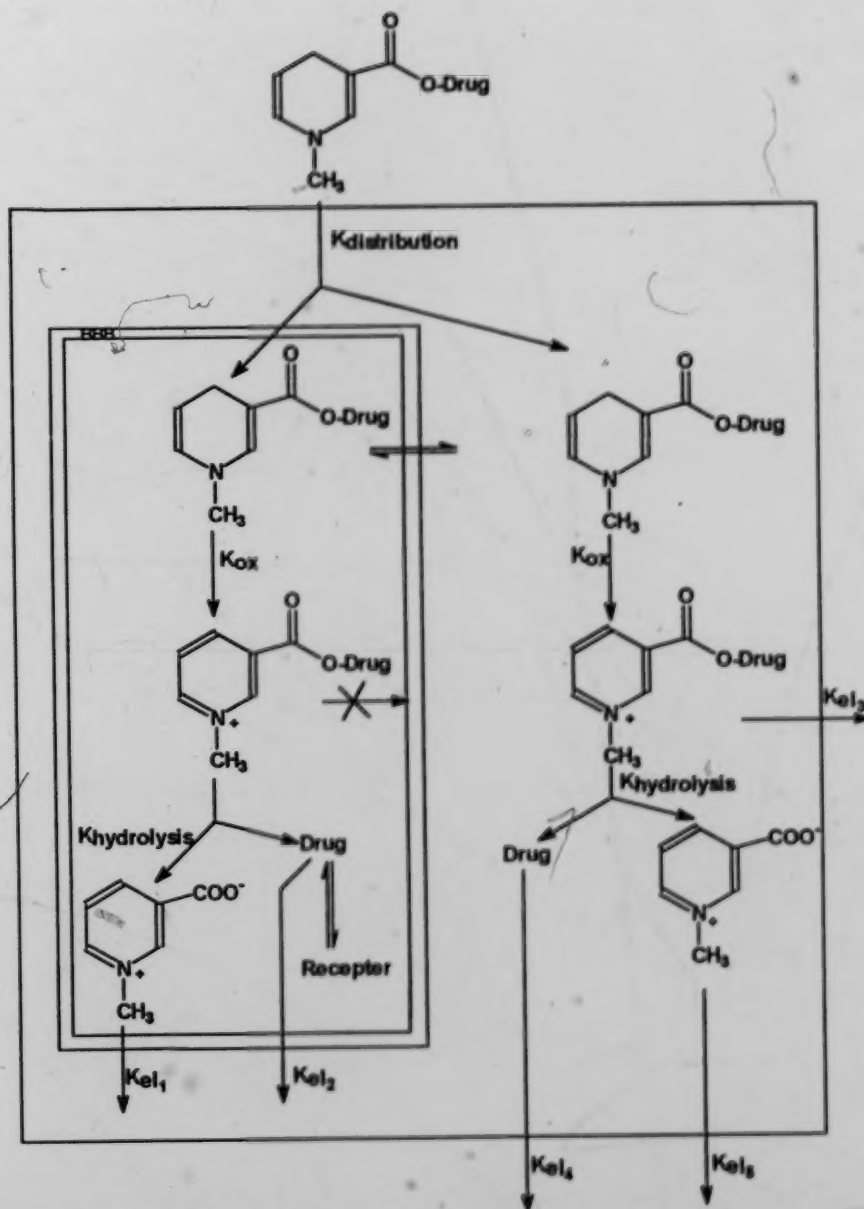


Figure 1.17. Central nervous system-enhanced delivery of drugs using the CDS approach (Bodor *et al.*, 1981a).

#### 1.5.2.2.1.4. Amino acid esters (19)

Amino acid (tyrosinyl, isoleucinyl, lysinyl, phenylalanyl, glutamyl) esters of AZT (6) were synthesized in an attempt to deliver AZT (6) more efficiently by an active transport system used for amino acids to target cells at higher concentrations than that which can be achieved by AZT (6) alone (Aggarwal *et al.*, 1990, Agrawal, 1991, Lupia *et al.*, 1993, Tadayoni *et al.*, 1993). Moreover, bone marrow progenitor cells may lack the amino acid active transport system (Vistica, 1980), which may lead to a reduced toxicity (see 19 in Figure 1.16).

#### 1.5.2.2.1.5. Fatty acid prodrugs (20-22)

Fatty acid nucleoside derivatives possess dramatically different physicochemical properties from the parent nucleoside compounds. The compounds are lipophilic with a high partition coefficient. The fatty acid moiety enhances drug penetration through membranes, is rapidly cleaved from the nucleoside by any of a variety of enzymes present in the cell cytoplasm. The deacylated compound could then be a substrate for phosphorylation needed to generate the active compound (Heiati *et al.*, 1997, Bibby *et al.*, 1996).

Ester prodrugs of zidovudine have been synthesized with aliphatic acids (sterically hindered acetic acids), and their enzymatic regeneration to zidovudine was investigated both *in vitro* and *in vivo* (Kawaguchi *et al.*, 1990) (see 20 in Figure 1.16).

Seki *et al.* (1990) synthesized five aliphatic esters (acetate, butyrate, hexanoate, octanoate, and decanoate) of AZT (6) and showed that these esters possessed an enhanced ability to permeate human skin relative to application of AZT (6) itself (see 20 in Figure 1.16).

Administration of vitamin A to retrovirally infected mice increased the number of activated macrophages and decreased death rate. A retinoic acid ester has been prepared by Agrawal (1991). This compound showed a substantial improvement in cellular uptake by H9 cells relative to the cellular uptake of AZT (6) (see 21 in Figure 1.16).

Horrobin *et al.* (1993, 1994) studied 5'-O-esters of AZT (6) with long chain (C:16 upwards) unsaturated fatty acids like linoleic acid (18:2,  $\Delta$ -9,12) and gamma-linoleic acid

(GLA) (18:3,  $\Delta$ -6,9,12) to enhance the transport of AZT (6) across lipid barriers, particularly from the gut into the lymph system, into cells from the extra-cellular fluid, or across the blood brain barrier. These compounds may be used percutaneously in topical preparations (see 22 in Figure 1.16).

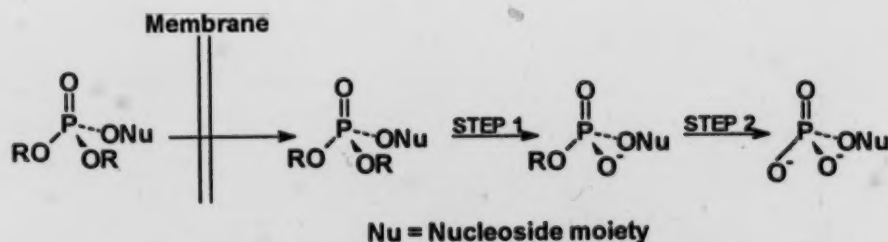
#### 1.5.2.2.2. Phospho derivatives (23-58)

With the aim of increasing AZT antiviral activity, several investigators have elaborated AZT into a variety of lipophilic phospho derivatives. A detailed discussion of their use is beyond the scope of this thesis. Accordingly, a short and tabular review is given here.

(1) Nucleotides cannot be used as chemotherapeutic agents because they are unable to cross cell membranes efficiently due to their polar nature. Moreover, they are readily dephosphorylated on cell surfaces and in extracellular fluids by non-specific phosphohydrolases. On the other hand, zidovudine and other ddNs do not reduce the amount of HIV isolated from peripheral mononuclear cells. Failure to clear the infectious virus from these cells may be due to inadequate inhibition of virus production by macrophages, a major reservoir of HIV infection (Lefebvre *et al.*, 1995a, b, Hostetler *et al.*, 1990b). To overcome these problems, various neutral 5'-nucleotide esters have been prepared in the hope that they penetrate cells by passive diffusion.

(2) In attempts to bypass the first, and limiting, phosphorylation step in the metabolic conversion of the ddN analog to its 5'-triphosphate derivative (ddN-TP), numerous prodrugs of the 5'-monophosphate (ddN-MP) types have been proposed, with the hope that these prodrugs would release the ddN-MP intracellularly. A variety of prodrugs with a phosphate linkage have been designed to deliver AZT (6) as its monophosphate, thereby overcoming the problem of inadequate monophosphorylation (Figure 1.18).

(3) The problem of glucuronidation and initial phosphorylation could be avoided by using the 5'-monophosphate of AZT. Since AZT-MP is dianionic at physiologic pH, it is unable to diffuse across cell membranes. Furthermore, the 5'-monophosphate is thought to be responsible for toxicity associated with AZT (6). One approach would be to prepare a lipophilic derivative of the monophosphate which will slowly release monophosphate in a controlled manner.



R = alkyl, aryl, diacyl glycerols, ethers, thioethers, amidoalkyls, carbohydrates,...

Figure 1.18. Bypassing kinase using masked phosphates.

Several groups have synthesized phosphate triester, phosphonate, phosphoroamidate and triphosphate derivatives of AZT (masked phosphate esters) as membrane-soluble depot forms of the bio-active nucleotide of AZT (6) (McGuigan *et al.*, 1991a, b, Freeman *et al.*, 1992, Desseaux *et al.*, 1993). These include nucleoside conjugates of simple dialkyl phosphate esters (McGuigan, 1990b, c), lactyl and glycolyl phosphate esters (McGuigan *et al.*, 1992a), various cholesteryl, alkyl, aryl, and diaryl phosphate derivatives (McGuigan *et al.*, 1992b, Sergheraert *et al.*, 1993, Thomson *et al.*, 1993, McGuigan *et al.*, 1994a, b, c), phosphorodiamidate derivatives (McGuigan *et al.*, 1991b), diacylglycerols, either monophosphate (Steim *et al.*, 1990, Hostetler, *et al.*, 1990b) or diphosphate (Hostetler, 1990b, van Wijk *et al.*, 1992a, b, Hostetler, *et al.*, 1992), glycosyl phosphotriesters of AZT (6) (Henin *et al.*, 1991), and those of either thioether or amidoalkylether lipids with monophosphate (Piantadosi *et al.*, 1991) and phosphonates (Piantadosi *et al.*, 1991). Some of the important examples are discussed here.

#### 1.5.2.2.1. Phosphate triesters (dialkyl or diarylphosphate esters) (23-28)

It has been found that simple dialkyl phosphotriester derivatives of AZT, and other nucleoside analogs, are inactive as anti-HIV agents. In contrast, haloalkyl and other lipophilic long chain phosphotriesters of AZT (24) showed comparable activity to that of AZT (6) (Figure 1.19) (McGuigan, *et al.*, 1990b, McGuigan *et al.*, 1990c). Introduction of halogen atoms into the alkyl (phosphate) chains enhanced anti-HIV activity. To some extent, the potential of the phosphate to undergo alkyl cleavage to release the free



nucleotide appeared to correlate with antiviral activity (McGuigan, Turner, 1994). The same group (McGuigan *et al.*, 1994b, 1994c) has prepared diaryl triester analogs of AZT (25, 26). *In vitro* evaluation revealed the compounds exhibit a pronounced and selective antiviral effect, the magnitude of which varied considerably with the nature of the aryl substituent. McGuigan *et al.* (1994c) reported that diaryl phosphate derivatives of AZT (25, 26) (Figure 1.19) are extremely poor inhibitors of viral replication in a thymidine kinase deficient cell line. One possibility is that the high lability of the aryl group results in rapid hydrolysis *in vitro* to release the nucleotide extracellularly. Thus, it is apparent that diaryl phosphates are only able to act as prodrugs to the free nucleoside. Due to poor membrane penetration by such polar species as AZT-MP, the product would only act following extracellular cleavage to the nucleoside (AZT, 6), followed by passive membrane penetration, and intracellular re-phosphorylation (McGuigan *et al.*, 1994c).

Novel mixed phosphate derivatives (27, 29) were designed by Bodor (1992). The mixed phosphate derivatives are designed to be cleaved *in vivo* in stages after they have reached the desired site of action. The first cleavage of the terminal ester moiety in (29) by an esterase would afford an inherently unstable intermediate of the type (30) which immediately and spontaneously releases  $R_2CHO$  and the negatively charged locked in intermediate (31) depicted above. With time, a second cleavage catalyzed by alkaline phosphatase would occur, releasing drug (D-OH, AZT). In the case of zidovudine, that is activated *in vivo* by phosphorylation, activation may occur system by enzymatic conversion of the locked-in intermediate with phosphokinase to the active triphosphate and/or by phosphorylation of drug itself after its release from the locked-in intermediate as described above. The identity of the groups must be carefully controlled so that the enzymatic cleavages occur in the proper order (Figure 1.20) (Bodor *et al.*, 1992).

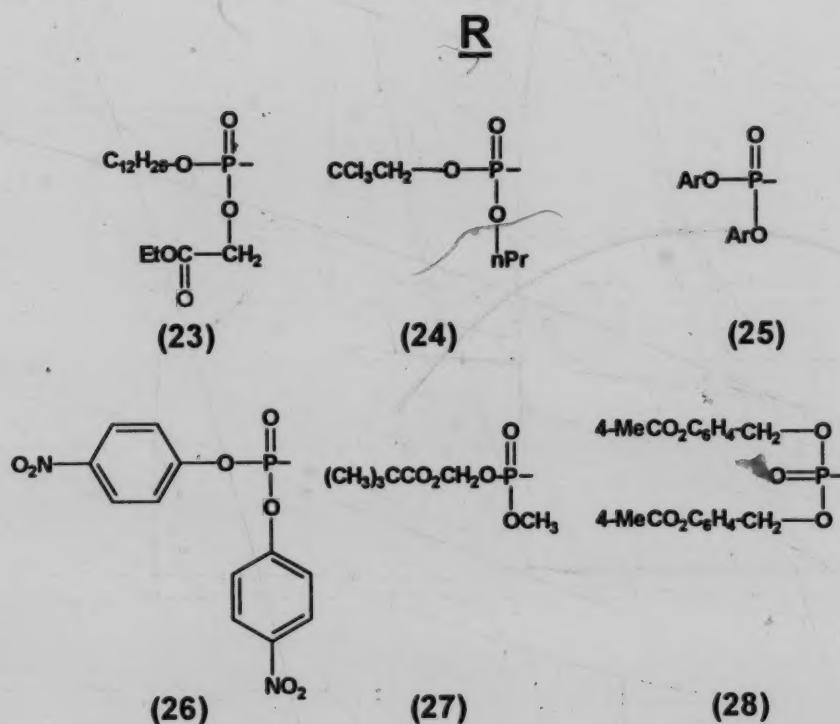


Figure 1.19. Phosphate triesters (dialkyl or diarylphosphate esters) (23-28).

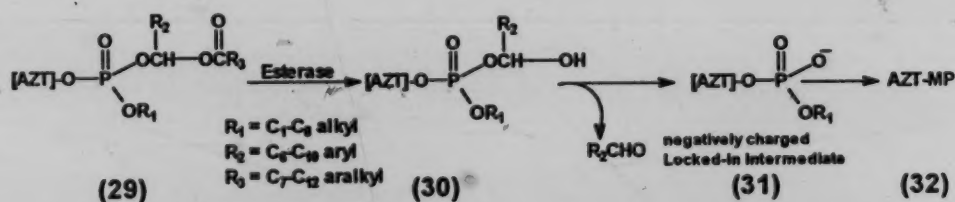


Figure 1.20. Bodor *et al.* (1992) approach for AZT delivery to brain

A similar approach was employed by Thomson *et al.* (1993) and Farquhar *et al.* (1990) using bis(4-acyloxybenzyl) esters (28, 33) of the 5'-monophosphate of AZT that after degradation produce anionic products (32, 35 and 36) that may be locked in the brain. The lipophilicity of the prodrug should facilitate passive diffusion across the BBB and into the brain. The mechanism of trapping is shown below. The success of this approach is dependent upon the design of an ester protecting group which will provide

suitable rates of hydrolysis. The problem of achieving this goal is highlighted by the case of simple phospho ester hydrolysis by P-O bond cleavage, where hydrolysis of the second group is typically  $1 \times 10^6$  times slower than the first. Metabolism of the prodrug to AZT (6) occurred predominantly within the cell (Figure 1.21) (Thomson *et al.*, 1993).

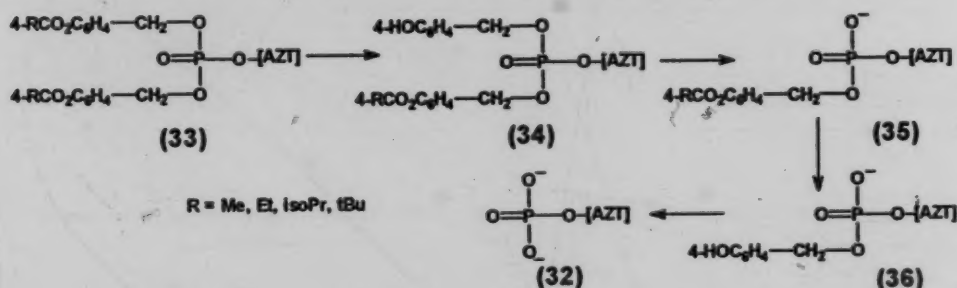


Figure 1.21. Thomson *et al.* (1993) approach for AZT delivery to brain.

#### 1.5.2.2.2. Phosphoroamidate derivatives (37, 38)

Some aryl phosphoroamidate derivatives of AZT (Figure 1.22) have been reported to exhibit anti-HIV activity in TK<sup>-</sup> cell lines. The data clearly demonstrated that the test compounds were efficient prodrugs to AZT-MP, partially circumventing the first activation step (phosphorylation of AZT (6) to its 5'-monophosphate) by thymidine kinase (McGuigan *et al.*, 1993). The mechanisms involved in the antiviral activity of these compounds remains unknown, although Valette *et al.* (1996) proposed that carboxyesterase and phosphodiesterase activation are involved in the activation process.

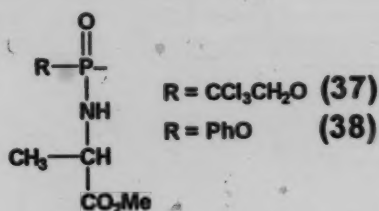


Figure 1.22. Phosphoroamidate derivatives.

#### 1.5.2.2.3. Carbohydrate derivatives (39-41)

A glycosyl phosphotriester prodrug of AZT was reported to provide good delivery of AZT (6) and AZT 5'-monophosphate (32) to the CNS. Therefore, glycosyl phosphotriesters (39-41) were evaluated as lipophilic prodrugs to AZT to increase brain uptake (Figure 1.23) (Namane *et al.*, 1992, Henin *et al.*, 1991). Different phosphate derivatives of AZT (6) coupled to a carbohydrate (D-glucose, D-mannose, and ethyl D-mannopyranoside) and a hexadecyl chain have been prepared for this approach. This system presents additional advantages over the esters discussed earlier (aliphatic and amino acid esters). 1) Hydrolysis of the prodrug yields the 5'-monophosphate which is the first metabolite of AZT anabolism by cellular kinases. This can be very important for macrophages which have lower levels of kinases, and for reducing the toxicity associated with AZT (6) because thymidine kinase is not needed. 2) The degradation products, a carbohydrate (glucose or mannose) and the lipidic alcohol (hexadecanol), do not contribute to toxicity. 3) Increased lipophilicity is expected because of the hexadecyl group. The solubility of the prodrugs in water allows oral administration due to the presence of glycosyl moiety. Studies suggested that the glycosyl phosphotriesters possessed a significantly improved pharmacokinetic profile of AZT-5'-phosphate in brain compared to that after AZT (6) dosing. AZT derivatives containing a polar carbohydrate and an hydrophobic hexadecyl chain retained their anti-HIV-1 activity and displayed high affinity for lipid membranes (Henin *et al.*, 1991, Schinazi *et al.*, 1992c).

Desseaux *et al.* (1993) synthesized lipophilic glycosyl phosphotriester derivatives of AZT (e.g. 41). Favorable transport of the nucleoside analog through the membrane was achieved. These compounds could be hydrolyzed *in situ* releasing the free nucleotide, that is the precursor of the active 5'-triphosphate. Compounds of this type that are combined with a tethered compound like the N,N,N'-trimethyldiamine (41) (Figure 1.23) are able to promote the degradation of RNA viruses.

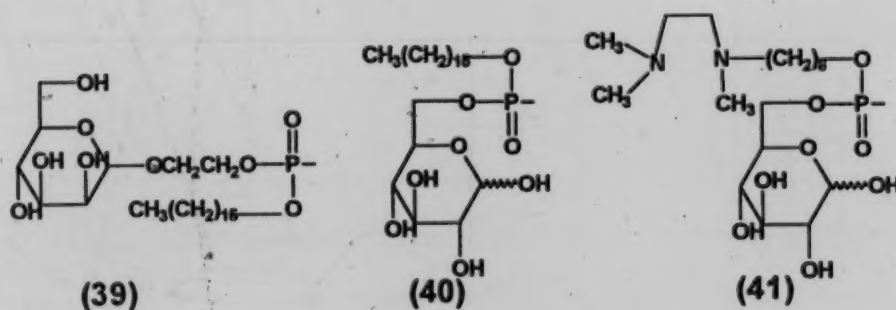
R

Figure 1.23. Carbohydrate derivatives (39-41).

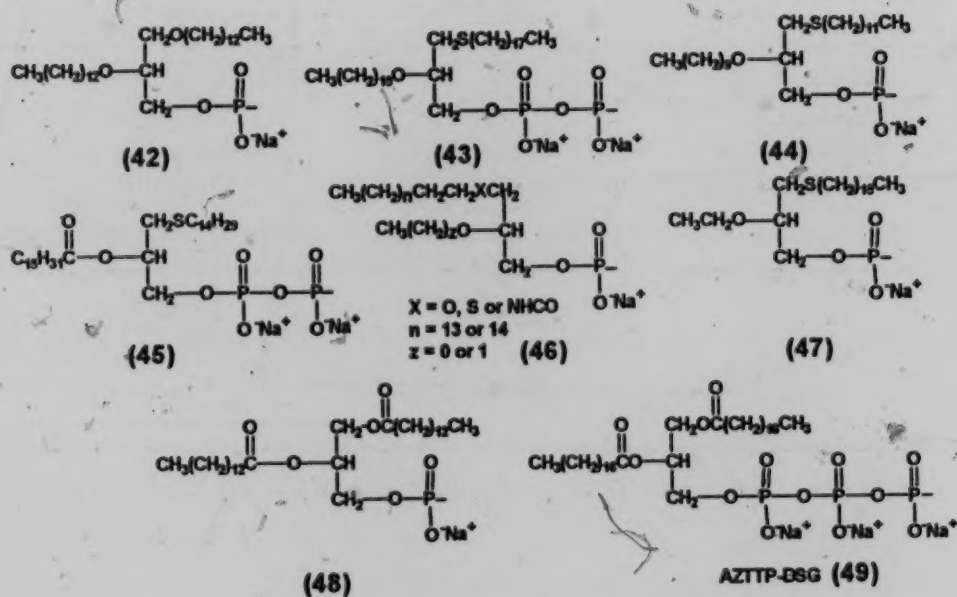
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Figure 1.24. Ether and ester lipids (42-49).

## 1.5.2.2.2.4. Ether lipids (42-47)

Ether lipids contain two active anti-HIV moieties comprised of a nucleoside analog and an ether lipid analog (EI), which are individually active, that have potent anti-HIV



activity into one molecule (42-47) (Figure 1.24). The two species released act at different stages of viral replication. The ELs are membrane interactive and the mechanisms for their action involve a shift in virus assembly from the plasma membrane to intracytoplasmic vacuoles and production of defective virus. The conjugate is a new prodrug of the nucleoside, that also may generate two antiretroviral agents, the 5'-triphosphate and the ether lipid analog, with different target sites. This combination could produce a synergistic action (Kucera *et al.*, 1990, Hong *et al.*, 1996b) since the nucleoside analog targets only RT.

Advantages of these derivatives are: 1) The ether lipid-nucleoside conjugates might allow a larger concentration of drug to enter the cell due to the lipophilic nature of the ether lipid. 2) Hydrolysis of the conjugate by cellular enzymes such as phosphatases might result in the slow release of nucleoside 5'-monophosphate into treated cells, possibly reducing resistance development to the nucleoside. 3) Degradative metabolism of the conjugate may release two active species (nucleoside 5'-monophosphate and ether lipid analogs), and 4) conjugates may provide cells with a depot form from which the antiviral drug can be released as the 5'-monophosphate species to bypass the required initial phosphorylation of free anti-HIV nucleosides (Hong *et al.*, 1996b).

Several groups have prepared different types of ether lipids (Puech *et al.*, 1993, Valette *et al.*, 1996, Lefebvre *et al.*, 1995a, b, Pompon *et al.*, 1994, Périgaud *et al.*, 1994, Meyer *et al.*, 1991, Piantadosi *et al.*, 1991, Tsotinis *et al.*, 1996, Hong *et al.*, 1996a, b, Hostetler *et al.*, 1995a, b).

In addition, ELs are not associated with myelosuppression or neuropathy shown by AZT (6). The mechanism of action of these ELs against HIV-1 involves a shift in virus assembly from the plasma membrane to intracytoplasmic vacuoles and the production of defective virus. ELs are lipophilic compounds that accumulate in the plasma membrane of T-lymphocytes and monocyte/macrophages and cross the host blood-brain (Piantadosi *et al.*, 1991, Hong *et al.*, 1996b).

Liponucleotides containing an ether linkage at the 2-position, and either a thioether, sulfoxide, or sulfone linkage at the 3-position, to the nucleoside component of AZT (6) or FLT (7) provided another approach used by Zilch *et al.* (1996) and Piantadosi *et al.*,

(1996) (see 43, 44 and 47 in Figure 1.24). AZT conjugates of amidoalkyl, oxyalkyl, and thioalkyl ether lipids (46) through phosphate linkages showed promising *in vitro* activity ( $EC_{50} = 0.02-1.56 \mu M$ ) and a 5-10 fold reduction in cell toxicity compared to AZT (6) alone (Piantadosi *et al.*, 1991).

#### 1.5.2.2.5. Nucleoside monophosphate, diphosphate and triphosphate diacylglycerols (ester lipids) (48, 49)

The antiviral activity of mono and polyunsaturated fatty acids has been known for over a decade. They are active against a number of enveloped viruses and thought to act in part by physical disruption of the virus particle. Therefore, polyunsaturated fatty acids or 12-methoxydodecanoic acid have been incorporated into anti-HIV nucleoside conjugates of ester lipids (Hong *et al.*, 1996a, b, Piantadosi *et al.*, 1991). Nucleoside monophosphate, diphosphate and triphosphate diacylglycerols have been designed to prodrugs of antineoplastic and antiviral nucleosides (Lambert *et al.*, 1995, Hostetler *et al.*, 1990). In thymidine kinase deficient mutant CEM cells, and other cell lines infected with human immunodeficiency virus (HIV), liponucleotides of AZT and other dideoxynucleotides were substantially more active than the corresponding parent drug (van Wijk *et al.*, 1992a). AZT conjugated to monophosphate diacylglycerol showed *in vitro* anti-HIV-1 activities comparable to that of the parent drug (6) (Steim *et al.*, 1990).

Hostetler and coworkers prepared phospholipid analogs of AZT (48) (Figure 1.24) and other antiviral nucleosides (liponucleotide prodrugs), which had anti-HIV-1 activity *in vitro* as liposome preparations (Hostetler *et al.*, 1991, 1992, 1993c), in attempts to improve AZT (6) therapy in HIV-1-infected human peripheral blood mononuclear cells (macrophages).

Mono- and diphosphate diglyceride conjugates of AZT are capable of inhibiting HIV replication *in vitro*, presumably as a result of releasing the respective nucleoside/nucleoside monophosphate from the lipid pro-drugs by intracellular-metabolizing enzymes that can be converted to triphosphate. Addition of the diacylglycerol diphosphate moiety to AZT (6) resulted in restoration of activity in TK<sup>-</sup>-CEM cells, suggesting direct intracellular metabolism to the respective monophosphate to

bypass the normally required thymidine kinase that is absent in TK<sup>-</sup> cells (van Wijk *et al.*, 1994).

#### 1.5.2.2.2.6. Dinucleoside phosphate derivatives (50-53)

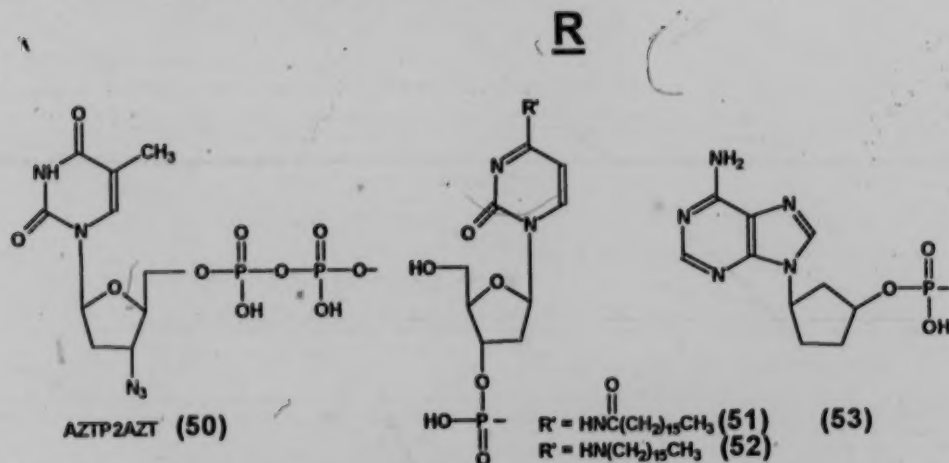


Figure 1.25. Dinucleoside phosphate derivatives (50-53).

More recently, new lipophilic alkyl/acyl dinucleoside phosphate derivatives of AZT (6) and other nucleosides have been synthesized (50-53) (Figure 1.25) (Velaquez *et al.*, 1995, Magnani *et al.*, 1996, Schwendener *et al.*, 1994, Meier *et al.*, 1995, Puech *et al.*, 1990, Thumann-Schweitzer *et al.*, 1996). These analogs are generally less active than AZT (6). To overcome the low ability of resting macrophages to phosphorylate antiviral deoxynucleosides, the homodinucleotide di(thymidine-3'-azido-2',3'-dideoxy-D-ribose)-5'-5'-p1-p2-pyrophosphate (AZTp2AZT, 50) was synthesized. Although it was a weak inhibitor of HIV reverse transcriptase, it showed antiviral ( $\text{EC}_{50} = 0.031 \mu\text{M}$ ) and cytotoxic ( $\text{IC}_{50} > 100 \mu\text{M}$ ) activities comparable to those of the parent AZT (6) ( $\text{EC}_{50} = 0.028 \mu\text{M}$ ,  $\text{IC}_{50} = 70 \mu\text{M}$ ) when added to cultures of a HTLV-1 transformed cell line. AZTp2AZT (50) encapsulated into erythrocytes was remarkably stable and upon phagocytosis allowed the targeted delivery of this impermeant drug to macrophages where metabolic activation occurs (Magnani *et al.*, 1996). The antiretroviral activity of this

compound is not directly elicited by the homodinucleotide analog, but most likely by the AZT-TP metabolite. Liposomal preparation of these newly synthesized dinucleoside phosphate derivatives may provide higher drug levels in plasma and virus-infected phagocytosing cells such as macrophages, monocytes and lymphocytes due to the natural affinity of liposomes for cells of the mononuclear phagocyte system.

The anti-HIV activity exhibited by the two lipophilic derivatives of AZT, *N*<sup>4</sup>-palmitoyl-2'-deoxyribocytidylyl-(3'-5')-3'-azido-2',3'-deoxythymidine(*N*<sup>4</sup>-palmitoylC-AZT, 51) and *N*<sup>4</sup>-hexadecyl-2'-deoxyribocytidylyl-(3'-5')-3'-azido-2',3'-deoxythymidine(*N*<sup>4</sup>-hexadecyl-AZT, 52) have been evaluated in comparison to AZT (6). Higher concentrations of these derivatives were required to obtain an antiviral effect similar to that obtained with free AZT (6). The higher concentration required may be due to the slow uptake of the micelles. Formation of other cellular metabolites of unknown structure and activity is conceivable and may further contribute to a delayed antiviral action (Schwendener *et al.*, 1994).

Cyclopentyl purine derivatives such as 53 show antiviral activity. A phosphate ester of these analogs with AZT (6) has been prepared to improve the efficacy of AZT (6) (Saksena *et al.*, 1990) (Figure 1.25).

#### 1.5.2.2.7. Others (54-58)

Calogeropoulou *et al.* (1995) reported the synthesis and biological evaluation of a series of new AZT (54) conjugates with alkyl and oxyalkyl ether phospholipids (Figure 1.26). In this group, the alkoxyethyl analog with a 12-carbon aliphatic chain possessed the most favorable therapeutic index.

Bonnaiffé *et al.* (1995) reported a new type of acyl nucleotides (55, 56) as potential nucleotide lipophilic prodrugs (Figure 1.26). In these compounds the lipophilic acyl moiety should enhance passive diffusion of the charged nucleotide through cell membranes. The mixed carboxylic phosphoric anhydride is expected to be hydrolyzed faster than the symmetrical phosphoric anhydride, thereby liberating the corresponding free nucleotide. When activities of acyl nucleotides were compared with their parent nucleotide, no differences in antiretroviral activity could be detected between acyl



nucleotides and their corresponding nucleotides at either 6 or 10 days after infection (Bonnaffé *et al.*, 1995, 1996).

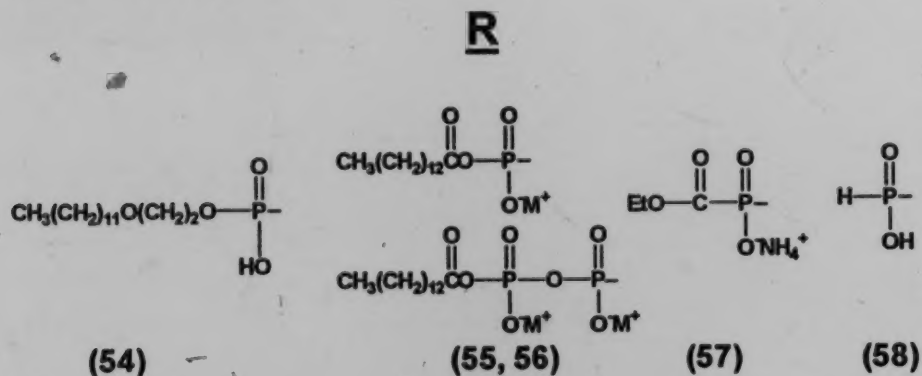


Figure 1.26. Other phosphate prodrugs of AZT.

The ammonium salt of 3'-azido-5'-(*O*-ethoxycarbonylphosphonyl)-3'-deoxythymidine (57), the prototype of a novel class of compounds incorporating two active antiretroviral agents, in this case AZT (6) and phosphoformic acid (PFA), within the same structure (Figure 1.26) was found to block viral replication at a 50% inhibitory concentration of 1  $\mu\text{M}$  (Rosowsky *et al.*, 1990). Other investigators have explored the potential of 5'-hydrogen phosphonates of anti-HIV nucleosides where it was concluded that their antiviral activity is due to release of the parent nucleoside (Gosselin *et al.*, 1993, McGuigan *et al.*, 1989, Krayevsky *et al.*, 1992). 5'-Hydrogen phosphonates of AZT (58) (see Figure 1.26) and FLT (59) (See Figure 1.27) exhibit potent anti-HIV-1 activity with selectivity indices similar to or better than those of their parent nucleosides. 5'-*O*-Hydrogen phosphonates, unlike highly acidic nucleoside 5'-phosphates (which cannot enter the cell), may penetrate the cell membrane due to their weakly acidic nature, and may be converted into the triphosphate analogs, pyrophosphorylhydrogenphosphonates, which then inhibit viral DNA synthesis catalyzed by RT, or oxidized to their corresponding phosphates and then further converted into the corresponding triphosphates in the cell. However, unlike AZT-MP, these phosphonates may not inhibit the dTTP formation until they are oxidized (Krayevsky *et al.*, 1992, Gosselin *et al.*, 1993, Freeman *et al.*, 1992).



## 1.5.3. Prodrugs of FLT (59-61)

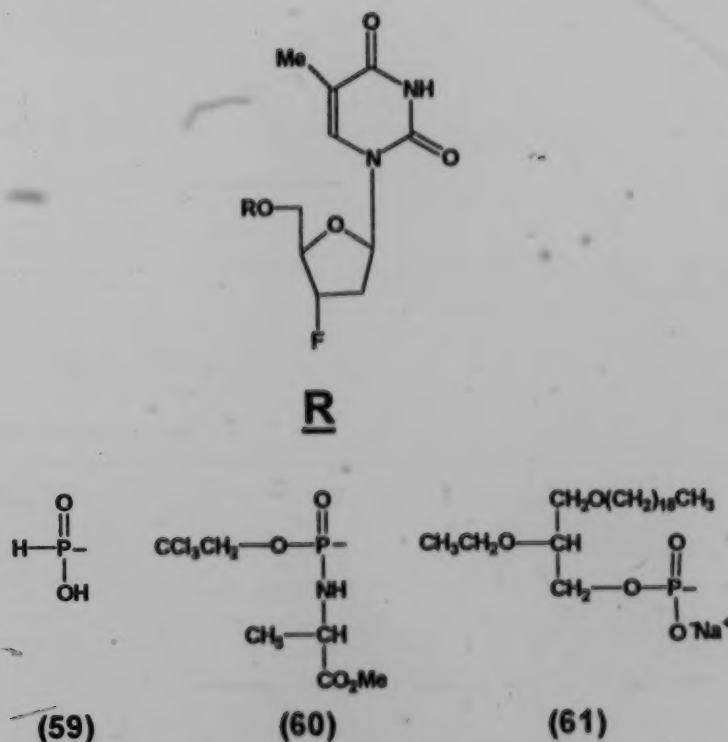


Figure 1.27. Some prodrugs of FLT.

The 5'-hydrogen phosphonate of FLT (59) showed potent anti-HIV-1 activity (Gosselin *et al.*, 1993). Several other approaches have been used for the synthesis of FLT prodrugs (McGuigan *et al.*, 1991b, Kumar *et al.*, 1994). Kumar *et al.* (1994) designed 5,6-dihydro prodrug derivatives of FLT (15) as mentioned before in Section 1.5.2.1. (Figure 1.15). McGuigan *et al.* (1991b) also prepared phosphate, phosphoramidate and phosphorodiamidate derivatives of FLT as potential prodrugs of the bioactive free nucleoside, since FLT (7) also suffers from an absolute dependence on host cell kinase mediated activation. Simple dialkyl phosphate triesters of AZT are inactive as antiviral agents (McGuigan *et al.*, 1990b), whereas bis(trihaloethyl)phosphates are active (see 24 in Figure 1.19) (McGuigan *et al.*, 1990c). The bis(trichloroethyl) phosphate analog of FLT

(60) was also active in the case of FLT (7) (McGuigan *et al.*, 1991b). The ether phospholipid derivative of FLT (61) has also been prepared by Rahim *et al.* (1991) (Figure 1.27).

#### 1.5.4. Myristoyl analog prodrugs (62-64)

3'-Deoxythymidine (3dT, 62) is a weakly active dideoxynucleoside in human immunodeficiency virus (HIV) infected cells because of its slow phosphorylation by cellular thymidine kinase. 3dT diphosphate dimyristoylglycerol (3dTDP-DMG, 63), a phospholipid prodrug, was found to be 18- to 50-fold more effective than 3dT in CEM and HT4-6C cells (Figure 1.28). In the CEM cell line, the selectivity index for 3dTDP-DMG (63) was 270 versus 48 for 3dT, an increase of 5.6 fold. The precise metabolic reason for the increased cellular activity may be due to increased cell uptake, and in part to bypassing the requirement for thymidine kinase (Hostetler *et al.*, 1992). Hong *et al.*, 1993 used a similar approach for 1- $\beta$ -D-arabinofuranosylcytosine conjugates (Ara-C) of thioether lipids using myristic acid as one of the fatty acids that were evaluated as anticancer agents. Myristoyl conjugates, which had convenient formulation properties, exhibited significant antitumor activity.

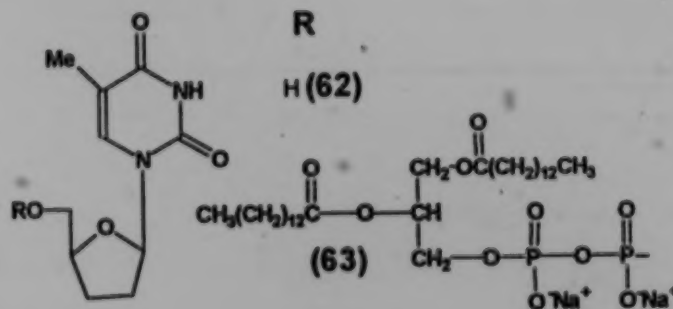


Figure 1.28. Myristoyl prodrugs of 3'-deoxythymidine.

*N*-myristoyl-(*S*)-phenylalanine (64) was introduced by Vincent *et al.* (1993) as an inhibitor of NMT for use in the treatment of cancer and retroviral infections (Figure 1.29). Novel fatty acid analogs that have from one to three heteroatoms such as oxygen, sulfur

or nitrogen in the fatty acid moiety have been synthesized by Nugent *et al.* (1997). The carboxy-terminus has been modified to form various amides, esters, ketones, alcohols, alcohol esters and nitriles. These compounds were effective as substrates for *N*-myristoyltransferase (NMT) and/or its acyl coenzyme, and as anti-viral and anti-fungal agents or prodrugs of such agents.

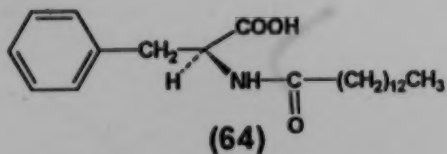


Figure 1.29. *N*-myristoyl-(*S*)-phenylalanine.

## 2. OBJECTIVES

Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) are two prominent infections which warrant the search for novel therapeutic targets. The main objective of this thesis project was to design, synthesize and pharmacologically evaluate a new class of pyrimidine nucleoside RT inhibitors prodrugs comprising a myristic acid analog and a nucleoside (dThd, AZT or FLT).

### 2.1. Myristic acid analogs

A variety of myristic acid analogs, possessing different physicochemical properties, that contain a halogen, azide, oxygen, sulfur or aromatic substituent have been investigated in this thesis. *In vitro* antifungal, and antiviral structure-activity relationships for a variety of myristic acid analogs including some novel compounds are described. This broad family of compounds permitted us to examine the effect of physicochemical properties, such as partition coefficient (Log P) and acidity ( $pK_a$ ), upon antifungal and antiviral activity.

Several mechanisms have been proposed for the antiviral and antifungal activities of myristic acid analogs. Inhibition of myristoyl CoA:protein *N*-myristoyltransferase (NMT) was discussed in detail previously as a probable and plausible mechanism based on substantial data in the literature (see section 1.4.2.3) and our results.

Myristoyl CoA:protein *N*-myristoyltransferase (NMT) is an attractive target for antiviral therapy to inhibit HIV-1 replication in AIDS patients and HBV replication in individuals infected by the hepatitis B virus, and fungal therapy to prevent opportunistic fungal infections caused by *C. neoformans* and *C. albicans*. *Aspergillus niger* contains saturated fatty acids such as myristic acid (Chattopadhyay *et al.*, 1985). Accordingly, this fungal infection is also expected to be sensitive to the incorporation of myristic acid analogs.

Myristic acid analogs that were investigated can be categorized into five groups:

- a) 4-oxa and 9-oxa analogs, b) 12-substituted dodecanoic acids, c) 11-substituted undecanoic acids, d) 2-substituted tetradecanoic acids, and e) 2-bromotetradecanoate esters.

## 2.2. Prodrug esters

Three categories of 5'-*O*-myristoyl analogue derivatives of dThd, AZT and FLT were investigated.

### 2.2.1. 5'-*O*-Myristoyl analogue derivatives of thymidine

The synthesis and biological properties of 5'-*O*-myristoyl ester derivatives of dThd are reported in this thesis. These analogs were designed to improve the delivery, and decrease the catabolism, of both dThd and the myristic acid analog in an attempt to improve their anti-HIV activity. *In vitro* anti-HIV I structure-activity relationships for these thymidine esters revealed unexpected anti-HIV-1 activities. Correlations between anti-HIV-1 activity of the fatty acids and their corresponding 5'-*O*-thymidine esters were investigated with respect to stability of the ester to porcine liver esterase and conformational features.

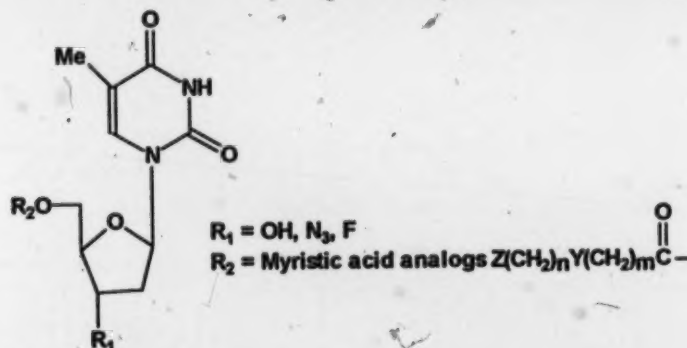


Figure 2.1. 5'-*O*-myristoyl ester derivatives of dThd, AZT and FLT.

### 2.2.2. 5'-*O*-Myristoyl analogue derivatives of AZT and FLT

Extensive efforts to synthesize lipophilic prodrugs to AZT (6) have not resulted so far in obtaining compounds with a clear-cut therapeutic efficacy. Although many prodrugs and other ddNs have been proposed, these research efforts have not provided a commercial product. Further research to identify prodrugs with clear advantages over AZT (6) and other nucleoside RT inhibitors is warranted. This thesis describes the synthesis of the 5'-*O*-ester conjugates of AZT and FLT (Figure 2.1), their stability, *in vitro* anti-HIV-1 activity, and preliminary pharmacokinetics of one candidate compound,



( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b), in mice. The major objectives of this project were to design a candidate prodrug to AZT in order to further improve the delivery characteristics and therapeutic efficacy of AZT, to examine the effect of a further increase in prodrug lipophilicity on brain and liver uptake, and to determine biodistribution of the prodrug. Fatty acids were covalently linked to anti-HIV nucleosides for several reasons. Fatty acids target a late step, and AZT targets an early step, in HIV-1 replication.

The advantages to this approach, since the agents act by different mechanisms of action, are: (1) Each individual compound exhibits anti-HIV activity and the degradative metabolism of the conjugate can release two active species that may result in an enhanced efficacy. The conjugate is not only a new prodrug but also may generate two antiretroviral agents, the nucleoside and the fatty acid analog, with different target sites. (2) Development of viral resistance to two active drugs most likely would occur at a slower rate than to either compound alone. (3) This combination of agents may reduce toxicity associated with nucleoside analogs like AZT. The two molecules act by different mechanisms of action and the fatty acids are not associated with bone marrow toxicity observed with AZT (6). Therefore, lower toxicity would be expected. (4) Fatty acid-AZT conjugates might allow a larger concentration of intact prodrug to enter the cell due to the lipophilic nature of the fatty acids. The prodrug is more lipophilic than the parent because the esters are more lipophilic compounds that accumulate in the plasma membrane of T-lymphocytes, monocytes/macrophages and will probably cross the BBB (increased brain uptake) in the host. A higher localization of AZT (6) in lymph nodes is expected that would prevent HIV infection in macrophages. (5) Hydrolysis of the conjugate by cellular enzymes as esterases might result in the slow release of AZT into treated cells, possibly increasing duration of action (prolonged plasma half-life). Extensive chemical modifications of the 5'-O-ester were performed to produce more lipophilic ester prodrugs to enhance brain and liver uptake, to sustain the release of AZT (6), and to prevent the fast clearance of the parent drugs. (6) Micelles formed from the conjugates could deliver the antiretroviral agents selectively to the HIV-1 infected cells. (7) A sustained release of AZT (6) or other nucleoside RT inhibitors would prevent spikes in the plasma

concentration, thereby reducing bone marrow toxicity since it is known that bone marrow toxicity is dose-related. Decreased delivery to the marrow, relative to blood levels, would also elevate the therapeutic index of these drugs. This could result in a requirement for less frequent dosages of prodrug compared to the individual prodrug moieties. (8) Another objective was to increase nucleoside uptake by the liver and to provide compositions for use as prodrugs which are metabolizable in the mammalian liver into a biologically active substance which is intended to exert its biological effect in the liver. Several ester analogs of FLT were prepared to reduce the toxicity and to increase its accumulation in hepatocytes.

The compounds prepared were evaluated by the U.S. National Cancer Institute Antiviral Evaluations Branch in an *in vitro* anti-HIV screen using HIV-1 infected CD4 lymphocytes (CEM cell line). The ability of a few compounds to inhibit hepatitis B virus (HBV) replication in chronically HBV-producing human liver cells (2.2.15 cells) was determined.

This thesis includes an evaluation of novel prodrugs of AZT, FLT and dThd with the goals listed below:

1. To determine the antifungal and antiviral activities of myristic acid analogs, their physicochemical properties and structure-activity relationships.
2. To evaluate anti-HIV and anti-HBV activities of 5'-O-myristoyl analogue derivatives of AZT and FLT.
3. To determine the physicochemical properties of 5'-O-myristoyl analogue derivatives of dThd, AZT and FLT and their correlation with antiviral activity.
4. To determine whether there is a correlation between *in vitro* hydrolysis of 5'-O-ester prodrugs and their *in vitro* anti-HIV activity.
5. To design a candidate prodrug for further biodistribution and pharmacokinetic studies.
6. To compare the pharmacokinetics of 3'-azido-3'-deoxythymidine (AZT, 6), and (±)-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) as a candidate prodrug in male Balb/c mice after iv administration.

### 3. RESULTS AND DISCUSSION

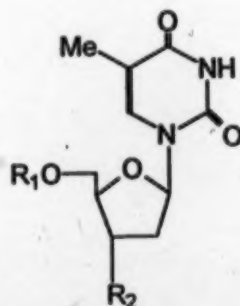
#### 3.1. Outline

The chemical structures of fatty acids (65a-71c) and the corresponding 5'-*O*-myristoyl analogue derivatives of dThd, AZT and FLT (81a-87, 91a-93) are shown in Tables 3.1 and 3.2. The synthesis of fatty acids (65a-71c) and 5'-*O*-fatty acyl of dThd, AZT and FLT (81a-93) will be discussed first followed by a detailed study of their biological evaluation (antifungal and antiviral activity), physicochemical properties and toxicity of the fatty acids. The antiviral activities of 5'-*O*-fatty acyl derivatives of dThd, AZT and FLT (81a-87, 91a-93) and their relationships to physicochemical properties and *in vitro* enzymatic and chemical stability are described next. The *in vivo* and pharmacokinetic study of one selected prodrug, ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-*O*-(2-bromomyristoyl)-thymidine (85b), is presented in final section.

Table 3.1. Chemical structures of fatty acids (65a-71c).

| No. | Structure                                                                            | No. | Structure                                             |
|-----|--------------------------------------------------------------------------------------|-----|-------------------------------------------------------|
| 65a | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH              | 67b | EtS(CH <sub>2</sub> ) <sub>10</sub> COOH              |
| 65b | N <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH | 67c | Br(CH <sub>2</sub> ) <sub>10</sub> COOH               |
| 65c | Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH              | 67d | 4-I-PhO(CH <sub>2</sub> ) <sub>10</sub> COOH          |
| 65d | Me(CH <sub>2</sub> ) <sub>4</sub> O(CH <sub>2</sub> ) <sub>7</sub> COOH              | 67e | PhO(CH <sub>2</sub> ) <sub>10</sub> COOH              |
| 65e | Me(CH <sub>2</sub> ) <sub>4</sub> S(CH <sub>2</sub> ) <sub>7</sub> COOH              | 67f | H <sub>2</sub> N(CH <sub>2</sub> ) <sub>10</sub> COOH |
| 66a | F(CH <sub>2</sub> ) <sub>11</sub> COOH                                               | 68a | Me(CH <sub>2</sub> ) <sub>11</sub> CH(F)COOH          |
| 66b | I(CH <sub>2</sub> ) <sub>11</sub> COOH                                               | 68b | Me(CH <sub>2</sub> ) <sub>11</sub> CH(OH)COOH         |
| 66c | Cl(CH <sub>2</sub> ) <sub>11</sub> COOH                                              | 68c | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOH         |
| 66d | Br(CH <sub>2</sub> ) <sub>11</sub> COOH                                              | 68d | Me(CH <sub>2</sub> ) <sub>11</sub> CH(I)COOH          |
| 66e | MeO(CH <sub>2</sub> ) <sub>11</sub> COOH                                             | 68e | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Cl)COOH         |
| 66f | EtS(CH <sub>2</sub> ) <sub>11</sub> COOH                                             | 69  | Ph(CH <sub>2</sub> ) <sub>9</sub> COOH                |
| 66g | Ph(CH <sub>2</sub> ) <sub>11</sub> COOH                                              | 70  | Me(CH <sub>2</sub> ) <sub>12</sub> COOH               |
| 66h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> COOH                                 | 71a | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOMe        |
| 66i | PhO(CH <sub>2</sub> ) <sub>11</sub> COOH                                             | 71b | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOEt        |
| 67a | MeO(CH <sub>2</sub> ) <sub>10</sub> COOH                                             | 71c | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOPr-i      |

**Table 3.2.** Chemical structures of 5'-O-fatty acyl derivatives of dThd, AZT and FLT (81a-87, 91a-93).



| No. | R <sub>1</sub>                                                                      | R <sub>2</sub> | No.      | R <sub>1</sub>                                                       | R <sub>2</sub> |
|-----|-------------------------------------------------------------------------------------|----------------|----------|----------------------------------------------------------------------|----------------|
| 81a | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                                 | OH             | 83i      | PhO(CH <sub>2</sub> ) <sub>11</sub> CO                               | N <sub>3</sub> |
| 81b | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO                           | OH             | 84a      | MeO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> |
| 81c | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                               | OH             | 84b      | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | N <sub>3</sub> |
| 81d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | OH             | 84c      | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                | N <sub>3</sub> |
| 81e | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO                | OH             | 84d      | 4-I-PhO(CH <sub>2</sub> ) <sub>10</sub> CO                           | N <sub>3</sub> |
| 81f | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(N <sub>3</sub> )CO              | OH             | 84e      | PhO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> |
| 81g | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                                  | OH             | 84f      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                   | N <sub>3</sub> |
| 81h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | OH             | 85a      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(F)CO             | N <sub>3</sub> |
| 82a | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO               | N <sub>3</sub> | 85b      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO            | N <sub>3</sub> |
| 82b | N <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO  | N <sub>3</sub> | 86       | Ph(CH <sub>2</sub> ) <sub>9</sub> CO                                 | N <sub>3</sub> |
| 82c | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO | N <sub>3</sub> | 87       | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | N <sub>3</sub> |
| 82d | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> O(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 91a      | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                | F              |
| 82e | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> S(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 91b      | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                               | F              |
| 83a | F(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 91c      | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO | F              |
| 83b | I(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 91d      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                   | F              |
| 83c | Cl(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 92       | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | F              |
| 83d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 93       | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | F              |
| 83e | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | N <sub>3</sub> | AZT (6)  | H                                                                    | N <sub>3</sub> |
| 83f | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO                | N <sub>3</sub> | FLT (7)  | H                                                                    | F              |
| 83g | Ph(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | dThd (8) | H                                                                    | OH             |
| 83h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | N <sub>3</sub> |          |                                                                      |                |

### 3.2. SYNTHESSES

#### 3.2.1. Synthesis of myristic acid analogs

The compounds that were investigated can be categorized into six groups:

- a) 4-Oxa, 9-oxa and 9-thia analogs:  $R(CH_2)_mX(CH_2)_nCOOH$  X  
 $R = Cl, n = 2, m = 8$  (65a), O  
 $R = N_3, n = 2, m = 8$  (65b), O  
 $R = Me, n = 2, m = 9$  (65c), O  
 $R = Me, n = 7, m = 4$  (65d), O  
 $R = Me, n = 7, m = 4$  (65e), S
- b) 12-Substituted dodecanoic acids:  $R(CH_2)_{11}COOH$   
 $R = F$  (66a),  $I$  (66b),  $Cl$  (66c),  
 $Br$  (66d),  $MeO$  (66e),  $EtS$  (66f),  
 $Ph$  (66g),  $N_3$  (66h),  $PhO$  (66i),  
 $OH$  (66j).
- c) 11-Substituted undecanoic acids:  $R(CH_2)_{10}COOH$   
 $R = MeO$  (67a),  $EtS$  (67b),  $Br$  (67c),  
 $4-I-PhO$  (67d),  $PhO$  (67e),  $NH_2$  (67f).
- d) 2-Substituted tetradecanoic acids:  $CH_3(CH_2)_{11}CH(R)COOH$   
 $R = F$  (68a),  $OH$  (68b),  $Br$  (68c),  
 $I$  (68d),  $Cl$  (68e).
- e) Others  $Ph(CH_2)_9COOH$  (69),  $CH_3(CH_2)_{11}COOH$  (70).
- f) 2-Bromotetradecanoate esters:  $CH_3(CH_2)_{11}CH(Br)COOR$   
 $R = Me$  (71a),  $Et$  (71b),  $i-Pr$  (71c).

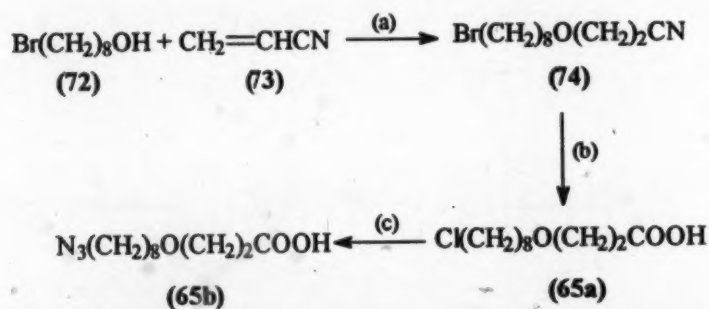
Methods that were reported previously were used to synthesize 4-oxatetradecanoic acid (65c) (Langner *et al.*, 1992), 9-oxatetradecanoic acid (65d) (Devadas *et al.*, 1992, Heuckeroth *et al.*, 1990), 9-thiatetradecanoic acid (65e) (Kishore *et al.*, 1991), 12-methoxydodecanoic acid (66e) (Gordon *et al.*, 1991, Nugent *et al.*, 1997), 12-thioethyldodecanoic acid (66f) (Gordon *et al.*, 1991), 12-



azidododecanoic acid (66h) (Adams *et al.*, 1992), 11-methoxyundecanoic acid (67a) (Gordon *et al.*, 1991, Nugent *et al.*, 1997), 11-thioethylundecanoic acid (67b) (Gordon *et al.*, 1991, Heuckeroth *et al.*, 1988), and (±)-2-fluorotetradecanoic acid (68a) (Paige *et al.*, 1990).

Improved synthetic methods have been developed for the synthesis of 12-fluorododecanoic acid (66a) and 12-chlorododecanoic acid (66c). 12-Chloro-4-oxadodecanoic acid (65a), 12-azido-4-oxadodecanoic acid (65b), 12-phenoxydodecanoic acid (66i), and 11-(4-iodophenoxy)undecanoic acid (67d) are new fatty acids (Parang *et al.*, 1996).

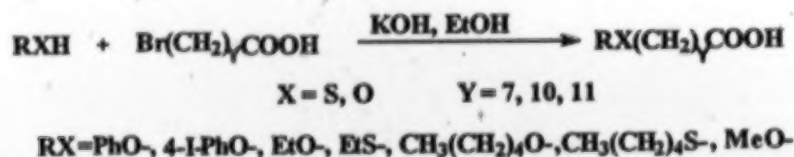
12-Chloro-4-oxadodecanoic acid (65a, 76%) was prepared by cyanoethylation of 8-bromooctanol (72) followed by hydrolysis of the cyano group to the carboxyl group as illustrated in Scheme 3.1. 12-Azido-4-oxadodecanoic acid (65b, 36%) was prepared by the addition of sodium azide to 4-chloro analog (65a) (Scheme 3.1).



Scheme 3.1. Synthesis of 12-chloro-4-oxadodecanoic acid (65a) and 12-azido-4-oxadodecanoic acid (65b); Reagents and conditions: (a) NaH, 55-60 °C, 3 h; (b) HCl, CH<sub>3</sub>COOH, 25 °C, 2 h. (c) NaN<sub>3</sub>, 60 °C, 8h.

Preparation of oxa- and thia- substituted fatty acids (65c-e, 66e-f, 66i, 67a-b, 67d-e) was effected using methods analogous to the preparation of mixed ethers by the Williamson synthesis. For example, an appropriate ω-bromo carboxylic acid or ester was reacted with an alcohol or an alkyl thiol to produce the oxa-substituted fatty acid ether or the thia-substituted fatty acid ether, respectively. This procedure can be illustrated by the

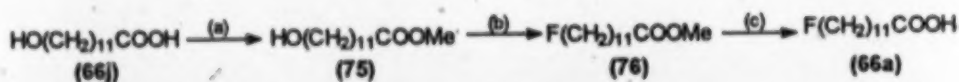
general reaction as illustrated below (Scheme 3.2):



**Scheme 3.2.** General procedure for the preparation of oxa- and thia- substituted myristic acid analogs.

11-Phenoxyundecanoic acid (**67e**) has been previously prepared by reaction of phenol with 11-bromoundecanoic acid (**67c**) in aqueous sodium hydroxide (Dieckman *et al.*, 1969). In this study, a similar method was used for the synthesis of 11-phenoxyundecanoic acid (**67e**, 68%), the previously unknown 12-phenoxydodecanoic acid (**66i**, 96%) and 11-(4-iodophenoxy)undecanoic acid (**67d**, 43%) in ethanol-potassium hydroxide (Parang *et al.*, 1996).

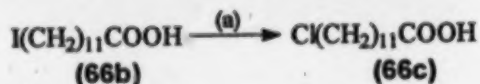
12-Fluorododecanoic acid (**66a**) was prepared previously using a tedious method in six steps from ethyl 11-bromoundecanoate (Pattison *et al.*, 1956). In the present study, 12-fluorododecanoic acid (**66a**) was prepared using a three step reaction that involved the elaboration of 12-hydroxydodecanoic acid (**66j**) to the methyl ester derivative (**75**) by reaction with trimethylsilyl chloride in methanol (Brook *et al.*, 1983). Subsequent reaction with diethylaminosulfur trifluoride (DAST), to replace the hydroxyl group by a fluorine substituent (Hudlicky *et al.*, 1988), and then acid hydrolysis of the methyl ester (**76**) yielded 12-fluorododecanoic acid (**66a**, 65%) (Scheme 3.3) (Parang *et al.*, 1996).



**Scheme 3.3.** Synthesis of 12-fluorododecanoic acid (**66a**); Reagents and conditions: (a) (CH<sub>3</sub>)<sub>3</sub>SiCl, CH<sub>3</sub>OH, 25 °C, 16 h; (b) DAST, benzene, 25 °C, 2 h; (c) HCl, dioxane, reflux, 2 h.

The method of Finkelstein was used to synthesize 12-iodododecanoic acid (**66b**) and ( $\pm$ )-2-iodotetradecanoic acid (**68d**) by reaction of the corresponding bromo analog (**66d**, **68c**) with sodium iodide in acetone (Peseckis *et al.*, 1993, Parang *et al.*, 1996).

The selective introduction of a chlorine substituent at the terminal methyl position of fatty acids is a challenging synthetic reaction. It was found that addition of concentrated nitric acid to a mixture of an alkyl iodide and hydrochloric acid resulted in an exothermic reaction that was accompanied by expulsion of elemental iodine to yield the corresponding alkyl chloride as the major product (Svetlakov *et al.*, 1969). This methodology was used for the successful preparation of 12-chlorododecanoic acid (**66c**) (Scheme 3.4) and ( $\pm$ )-2-chlorotetradecanoic acid (**68e**).



**Scheme 3.4.** Synthesis of 12-chlorododecanoic acid (**66c**); Reagents and conditions: (a)  $\text{HNO}_3$ ,  $\text{HCl}$ , 15-20 °C, 2 h.

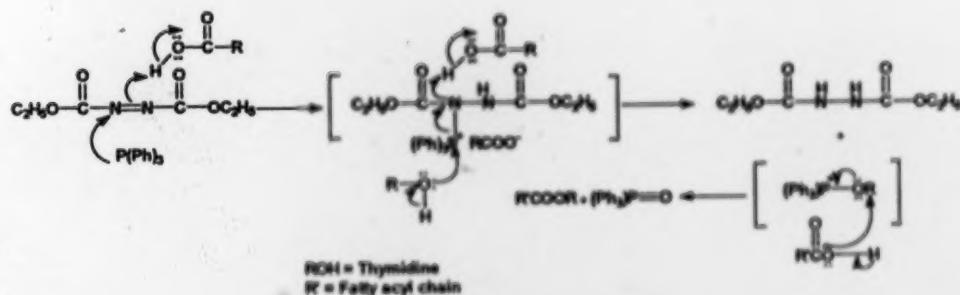
Phenyl analogs of fatty acids (**66g**, **69**) were synthesized using a Friedel Craft alkylation reaction (Scheme 3.5). 12-Phenyldodecanoic acid (**66g**, 49%) was prepared using the four step reaction sequence reported by Goodman *et al.* (1984). An alternate method reported by Eisenhut *et al.* (1988) was employed for the preparation of 10-phenyldecanoic acid (**69**, 49%) and 12-phenyldodecanoic acid (**66g**, 23%) (Scheme 3.5).

(±)-Methyl 2-bromotetradecanoate (71a, 92%), (±)-ethyl 2-bromotetradecanoate (71b, 72%), and (±)-isopropyl 2-bromotetradecanoate (71c, 92%) were prepared by esterification of (±)-2-bromotetradecanoic acid (68c) with trimethylsilyl chloride in either MeOH, EtOH or i-PrOH, respectively (Peseckis *et al.*, 1993).

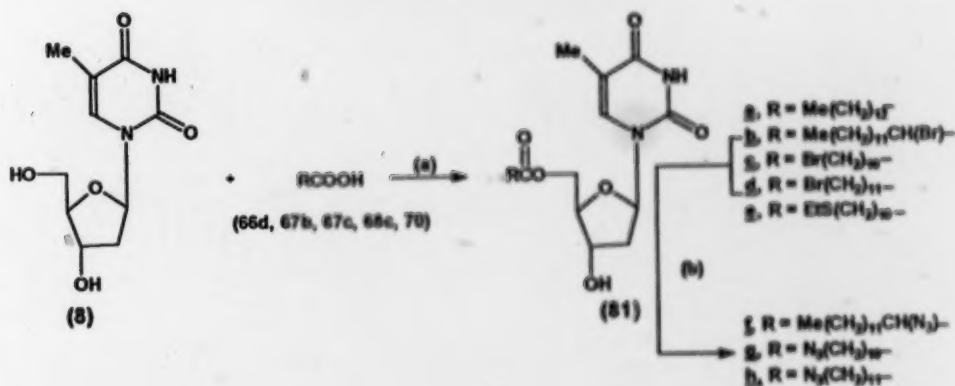
Various strategies were used for the esterification of 5'-OH group in AZT (6), FLT (7) and dThd (8). The acylated derivatives of these nucleosides were synthesized by reacting the pyrimidine nucleoside with an activated carboxylic acid. An activated carboxylic acid is one that has been treated with appropriate reagents to render its carboxylate carbon more susceptible to nucleophilic attack than is the case with the original carboxylic acid.

The Mitsunobu reaction (Mitsunobu and Yamada, 1967) (Scheme 3.6) was used for direct 5'-*O*-acylation of thymidine (dThd) (8). Direct acylation of dThd (8) was effected by adding a solution of the respective myristic acid analog (66d, 67b, 67c, 68c and 70) and diethyl azodicarboxylate (DEAD) in dioxane (molar ratio of 1:1:1) to a solution of (8) and triphenyl phosphine in dioxane at 60 °C (see Scheme 3.7). Therefore, a series of 5'-*O*-acyl derivatives of thymidine (dThd) were prepared (81a-e) by direct acylation of

thymidine using the Mitsunobu reaction. Further treatment of the bromo analogs **81b-d** with sodium azide yielded the azido ester analogs **81f-h** (see Scheme 3.7). The 5'-*O*-acylated nucleosides **81a-h** were obtained in 16-92% yields. Chemical structures of the products were confirmed by NMR spectroscopy. The structures of the 5'-*O*-ester derivatives of thymidine **81a-h** are depicted in Table 3.2.



Scheme 3.6. Mitsunobu reaction.



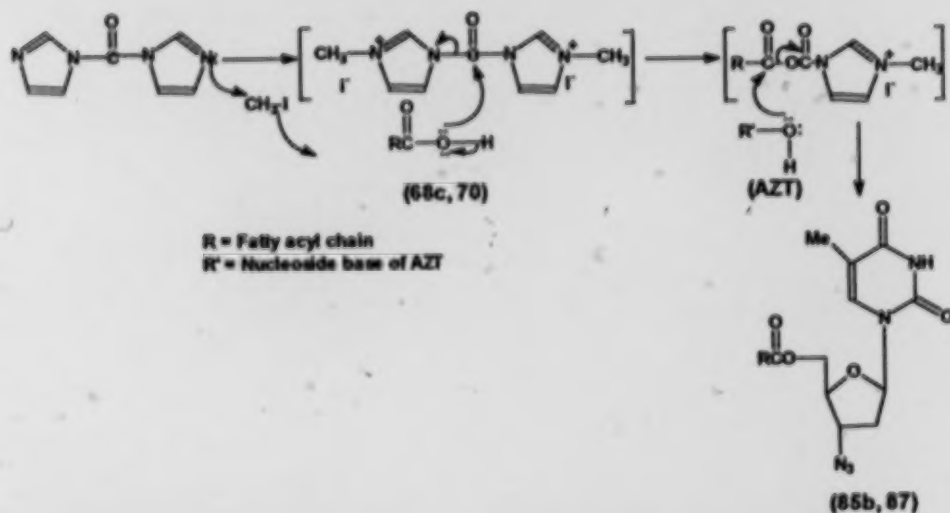
Scheme 3.7. Synthesis of 5'-*O*-myristoyl analogue derivatives of thymidine (**81a-h**); Reagents and conditions: (a) Diethylazodicarboxylate (DEAD), P(Ph)<sub>3</sub>, dioxane, 60 °C, 3 h. (b) NaN<sub>3</sub>, DMF, 8 h.



### 3.2.2.2. Synthesis of 5'-O-myristoyl analogue derivatives of AZT and FLT (82a-87, 91a-93)

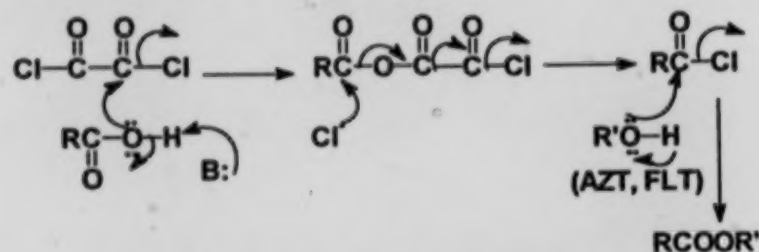
3'-Azido-3'-deoxythymidine (AZT, 6) and 3'-fluoro-3'-deoxythymidine (FLT, 7) were prepared using the literature procedure described by Czernecki *et al.*, (1991) and Herdewijn *et al.*, 1987), respectively. The Mitsunobu reaction (Mitsunobu and Yamada, 1967) can not be used for AZT esterification directly since azides are easily reduced to primary amines by a number of reducing agents such as triphenylphosphine (Staundinger reaction) (Meyer *et al.*, 1919). Several other approaches were used for the synthesis of various 5'-O-myristoyl analogs.

1. An improved and convenient one-pot synthesis of esters (Kamijo *et al.*, 1984) from carboxylic acids using 1,1'-carbonyldiimidazole (CDI) in the presence of a reactive halide was used for the synthesis of 2-bromomyristoyl (85b, 44%) and myristoyl (87, 64%) analogs. Thus, CDI was treated with a reactive halide such as methyl iodide (2 eq) in acetonitrile, and then the carboxylic acid (1 eq) was added to the solution followed by addition of AZT to afford the corresponding esters in high yields (Scheme 3.8).



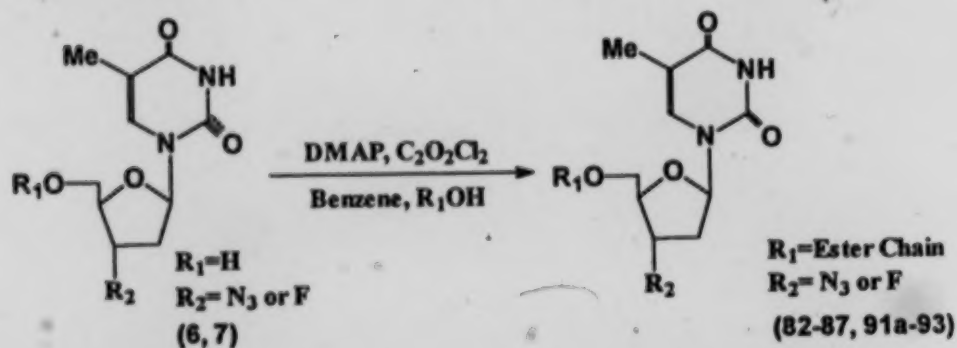
Scheme 3.8. Synthesis of 85b and 87 using 1,1'-carbonyl diimidazole and methyl iodide.

2. The 5'-OH function of AZT (6) and FLT (7) was esterified with various myristic acid derivatives in the presence of oxalyl chloride and 4-(dimethylamino)pyridine (DMAP). The esterification reaction in the case of AZT required 4-(dimethylamino)pyridine (DMAP) as a catalyst. The esterification reactions in the presence of catalytic amounts of DMAP remained incomplete even after long periods of reaction time. However, addition of DMAP in the presence of oxalyl chloride produced the corresponding esters (82-87) in approximately 50-70% yields. The fatty acid (RCOOH) was therefore reacted with oxalyl chloride in benzene prior to its reaction with AZT in the presence of approximately one equivalent of DMAP. A similar method was used for 5'-O-esterification of FLT (7) (Schemes 3.9 and 3.10).



R = fatty acyl chain R' = nucleoside base for AZT and FLT

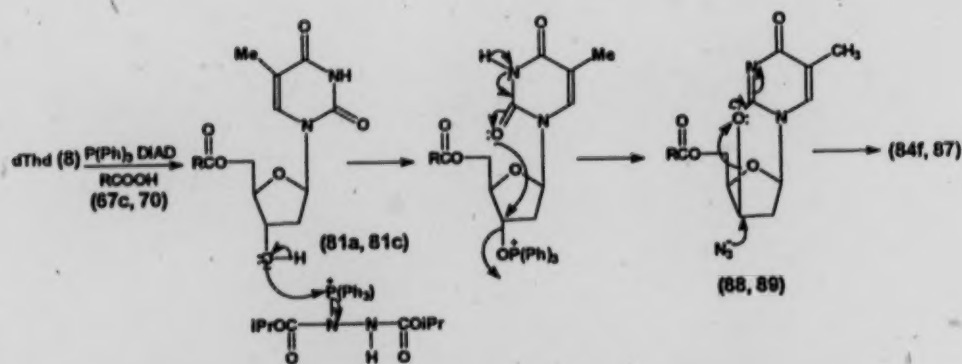
Scheme 3.9. General mechanism for 5'-O-esterification of AZT and FLT using oxalyl chloride and DMAP.



Scheme 3.10. General reaction for the preparation of 5'-O-myristoyl analogs of AZT (82-87) and FLT (91a-93).

3. 2,3'-Anhydro-1-(5'-*O*-myristoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (**88**) and 2,3'-anhydro-1-(5'-*O*-11-bromoundecanoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (**89**) can be prepared from thymidine (**8**) by a one-pot transformation involving a tandem Mitsunobu reaction (Czernecki *et al.*, 1991). A solution of thymidine (**8**) (4.1 mmol) in dimethylformamide was treated with diisopropyl azodicarboxylate (DIAD, 6.2 mmol) in the presence of triphenylphosphine ( $\text{Ph}_3\text{P}$ , 6.2 mmol) and the desired fatty acid (4.1 mmol) at room temperature. It was more convenient to allow the transformation to proceed to the 2,3'-anhydro derivative (**88**, **89**) by a further addition of triphenylphosphine (6.2 mmol), and DIAD (6.2 mmol), rather than isolating the intermediate 5'-ester. After the reaction mixture was stirred for 30 min, the cyclization was complete. Therefore, when the reaction was carried out using excess triphenylphosphine, it could be used to synthesize 2,3'-anhydrothymidine-5'-*O*-myristoyl analogue derivatives (**88**, **89**) from thymidine (**8**), and diisopropyl azodicarboxylate (DIAD) or DEAD (Scheme 3.11).

The ring opening of the 2,3'-anhydro derivative (0.85 mmol) was cleanly achieved with sodium azide (2.1 mmol) in dimethylformamide at 60 °C to yield **84f** (87%) and **87** (52%). No cleavage of the 5'-ester function was observed under these conditions (Scheme 3.11).



Scheme 3.11. Synthesis of **84f** and **87** using the Mitsunobu reaction.

### 3.3. Biological activity of myristic acid analogs and 5'-O-myristoyl analogs of dThd, AZT and FLT

The biological evaluation of prodrugs to anti-HIV-1 nucleosides are reported in this thesis. Briefly, the compounds investigated and their potential therapeutic applications are based on the utilization of esters of the biologically active compounds AZT and FLT as prodrugs. The objective was to increase uptake of the ester prodrug into cells and which in turn would increase the amount of AZT or FLT localized in cells following hydrolysis of the ester. Myristic acid analogs possessing altered physical-chemical properties, have recently been shown to exhibit antiviral and antifungal properties. Membrane interactive, fatty acid analogs are ideally suited for combination chemotherapy with DNA-interactive anti-HIV nucleoside analogs. The ester combination of a fatty acid analog and AZT produces an apparent synergistic effect, suppressing HIV-1 replication. The linking of a myristic acid analog to AZT via an ester moiety appears to enhance cellular permeability to AZT, and in some cases may possess further beneficial effects with respect to reduced toxicity and enhanced antiviral activity. Depending on the level of esterase activity and its ability to cleave the prodrug, a slow rate of formation or slow release of the active drug(s) from the prodrug may be achieved. The antifungal and antiviral activities, and physicochemical properties of myristic acid analogs will be described initially. The antiviral activity, cytotoxicity and physicochemical properties of the ester prodrugs and a pharmacokinetic study of one selected ester prodrug will be discussed later.

#### 3.3.1. Myristic acid analogs (65a-71c)

##### 3.3.1.1. Biological activity of myristic acid analogs

##### 3.3.1.1.1. *In vitro* antifungal activity of myristic acid analogs

A group of myristic acid analogs were investigated to develop antifungal structure-activity relationships and to determine the effect of physicochemical properties such as partition coefficient and acidity upon antifungal activity. A large difference in antifungal activities was observed for the myristoyl analogs investigated.

The *in vitro* antifungal test results for the fatty acids (65a, 65c, 65d, 66a-68e, 69,

70) and myristate esters 71a-c are presented in Table 3.3.

#### 3.3.1.1.1. Oxa and thio ethers

Introduction of an heteroatom such as oxygen or sulfur into the myristoyl moiety results in loss of a site for hydrophobic interaction and creates a potential hydrogen bond acceptor (Kishore *et al.*, 1993). Some oxa-substituted analogs such as 11-methoxyundecanoic acid (67a) and the thio-substituted analogs (66f, 67b) were only weak antifungals. This is in agreement with *in vitro* NMT assay data which show that they are poor NMT substrates (Kishore *et al.*, 1993).

4-Oxatetradecanoic acid (65c), which is incorporated into *C. neoformans* Arf, is reported to be fungicidal at 0.3 mM. Langer *et al.*, reported that 4-oxatetradecanoic acid (65c) produced an approximately 10-100-fold reduction in the viability of *C. albicans* and *S. cerevisiae* at a 0.3-0.5 mM concentration (Langner *et al.*, 1992). The oxa- (65c, 65d, 66e, 67a) and thia- (66f, 67b) myristic acid analogs generally exhibited medium antifungal activity. The 4-oxa analog (65c) exhibited potent activity against *C. neoformans* (MIC = 0.17 mM) that is comparable to reported data. Myristic acid analogs with an oxygen atom at position 9 in the fatty acid backbone are good NMT substrates. Detailed kinetic studies have shown that when 9-oxatetradecanoic acid (65d) is added to the coupled *in vitro* assay system, the calculated  $K_m$  of the peptide increases only about 2-fold compared to myristic acid (70), but the rate of acyl-peptide formation is slightly higher, producing a peptide catalytic efficiency 70% of that for myristic acid (70) (Heuckeroth *et al.*, 1990). The *in vitro* anti-fungal data acquired indicate that 9-oxatetradecanoic acid (65d) is relatively active agent only against *C. neoformans* (MIC = 0.04 mM) (Table 3.3). The 4-oxa (65c) and 9-oxa (65d) derivatives were active against *C. neoformans* (MIC = 0.04-0.17 mM) and exhibited moderate activity against *S. cerevisiae* (0.34 mM) and *A. niger* (0.34-0.68 mM). In contrast, 12-chloro-4-oxatetradecanoic acid (65a) was an inactive antifungal agent.



### 3.3.1.1.1.2. 12-Halo-substituted fatty acids

12-Substituted analogs 66a-h were active against *C. neoformans* (0.04-0.18 mM), but they exhibited weak activity against *C. albicans* (1.9- > 5 mM). Compounds 66b and 66d were active against *A. niger* at a concentration < 0.04 mM. Other halogenated analogs such as 11-bromoundecanoic acid (67c), 12-iodododecanoic acid (66b), 12-bromododecanoic acid (66d), and 12-chlorododecanoic acid (66c) were active against *C. neoformans* and *S. cerevisiae*. 12-Fluorododecanoic acid (66a) was a much less active antifungal agent compared to the iodo (66b), chloro (66c) and bromo (66d) analogs against *A. niger* (Table 3.3). A plausible explanation for the differences in their relative potency may be their relative affinities for the binding site of NMT. It has been suggested that specific amino acid residues present in NMT bind to specific parts of the fatty acid backbone and that the fatty acyl CoA may be rigidly bound by NMT. It is thought that the acyl-CoA binding site possesses a complex sensor that measures the distance between the carboxyl and the omega end of the fatty acid and the steric volume at the omega terminus (Kishore *et al.*, 1991).

### 3.3.1.1.1.3. Phenyl substituted fatty acids

An additional study was carried out to assess whether antifungal activity correlates with the presence and position of an aromatic ring or a heteroatom. Phenyl-substituted derivatives generally exhibited weak antifungal activity. For example, 10-phenyldecanoic acid (69) was equally active against *C. neoformans* and *S. cerevisiae* (MIC = 0.16 mM), but it was only weakly active against *A. niger* and *C. albicans*. 12-Phenyldodecanoic acid (66g) was found to be a potent antifungal agent only against *C. neoformans* (MIC = 0.04 mM). It has been suggested that the conversion of 10-phenyldecanoic acid (69) to its acyl-CoA derivative produces a 10-fold increase in peptide catalytic efficiency relative to the nonanoic or undecanoic acid derivatives. The  $K_m$  for 10-phenyldecanoyl-CoA (1.2  $\mu$ M) is comparable to that of myristoyl-CoA (3.8  $\mu$ M) (Kishore *et al.*, 1991). The antifungal activity exhibited by myristic acid analogs is not affected significantly by oxygen substitution in the chain or by

placement of an iodine substituent at the *para*-position of a phenyl ring (66i, 67e, 67d). Kishore *et al.*, (1991) proposed that compounds having a terminal phenyl moiety should not fit into the postulated conical receptor and therefore should exhibit weaker activity than the analogs having terminal methyl groups. Our *in vitro* antifungal data for 66g, 66i, 67d and 67e confirm this postulation since these compounds generally exhibited weaker antifungal activity than 69 (Table 3.3). This may be due to the enzyme's inability to accommodate the longer fatty acids at the conical binding site. The steric volume of substituents is likely another important factor that influences the binding affinity of the myristic acid analog to the NMT binding site.

#### 3.3.1.1.4. 2-Substituted tetradecanoic acids

The antifungal activities of the 2-substituted tetradecanoic acids 68a-e against *C. neoformans*, *A. niger*, *C. albicans*, and *S. cerevisiae* were also determined. Compounds 68c-e were active against all four fungi. The 2-bromo analog 68c was the most potent antifungal fatty acid tested with antifungal MICs of 0.02 mM, 0.01 mM, 0.04 mM and < 0.04 mM, respectively, against *C. neoformans*, *S. cerevisiae*, *C. albicans* and *A. Niger* (Table 3.3) in RPMI 1640 growth medium. It has been reported that (±)-2-bromotetradecanoic acid (68c) does not exhibit antifungal activity against *A. niger* at pH 4.0 and pH 5.6 in Sabouraud dextrose agar after 5 days at 28 °C (Gershon *et al.*, 1973). In this study the culture medium RPMI 1640 (pH = 7) was used to test 68c against *A. niger* (MIC < 0.04 mM). At pH = 7, the concentration of the ionized form is 8700-fold greater than the concentration of the unionized form of this carboxylic acid, whereas this ratio is only 347-fold larger at pH = 5.6. Therefore, the difference in antifungal activity between the reported and current studies may be due to differences in the pH of the culture media used in these experiments. Other 2-substituted analogs such as (±)-2-fluorotetradecanoic acid (68a) and (±)-2-hydroxydodecanoic acid (68b) exhibited very weak antifungal activity. These results would be expected if the mechanism of action involved fungal peptide alkylation at C-2 of the myristoyl analog, since bromine is a much better leaving group than fluorine

or hydroxyl substituents. This alkylation mechanism could be confirmed in part by testing ( $\pm$ )-2-iodotetradecanoic acid (**68d**) and ( $\pm$ )-2-chlorotetradecanoic acid (**68e**). Since iodine is a better leaving group than bromine, and chlorine is a less effective leaving group than bromine, one would predict that ( $\pm$ )-2-iodotetradecanoic acid (**68d**) would be a more potent antifungal agent than the 2-bromo analog **68c**, whereas ( $\pm$ )-2-chlorotetradecanoic acid (**68e**) should be a less active antifungal agent than the 2-bromo analog **68c**. The antifungal activities of the 2-iodo- (**68d**) and 2-chloro- (**68e**) substituted analogs were not significantly different than that of the ( $\pm$ )-2-bromotetradecanoic acid (**68c**), which suggests that factors other than displacement of the C-2 halogen substituent are determinants of antifungal activity.

The close proximity of the C-2 halogen substituent or the 4-oxa moiety to the carboxylic acid group increases the ability of the COOH to undergo dissociation (due to the electronegative effect of halogen or oxygen). The  $pK_a$  of the carboxylic acid moiety could be an important factor in myristoylation of fungal peptides, since the weakly active antifungal 2-substituted fatty acids **68a** ( $pK_a = 2.83$ ) and **68b** ( $pK_a = 4.01$ ) have a lower or higher calculated  $pK_a$  than the more active antifungal analogs **68c-e** ( $pK_a$  range = 2.99-3.30) (Table 3.3). Binding of the fatty acid to NMT is one of the first steps in myristoylation of a peptide that requires an active carboxylic acid moiety. Three alkyl esters of ( $\pm$ )-2-bromotetradecanoic acid (**68c**) were synthesized to determine the role of the carboxylic acid moiety with respect to antifungal activity. The differences in lipophilicity and the size of the alkyl ester substituent present in ( $\pm$ )-methyl 2-bromotetradecanoate (**71a**), ( $\pm$ )-ethyl 2-bromotetradecanoate (**71b**) and ( $\pm$ )-isopropyl 2-bromotetradecanoate (**71c**) could provide a correlation of antifungal activity with physicochemical properties. All three esters exhibited low antifungal activity against *C. neoformans*, *A. niger*, *C. albicans* and *S. cerevisiae*, suggesting that the carboxylic acid substituent is required for antifungal activity and that inhibition

Table 3.3. Structures, physicochemical properties and antifungal activity (MIC values, mM)<sup>a</sup> against *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Aspergillus niger* for fatty acids and esters.

| Structure                                                                     | Log P <sup>b</sup> | Log D <sub>7.0</sub> <sup>c</sup> | pKa <sup>d</sup> | <i>C. albicans</i><br>ATCC 14053 | <i>C. neoformans</i><br>KF-23 | <i>S. cerevisiae</i><br>PLM 454 | <i>A. niger</i><br>PLM 1140 |
|-------------------------------------------------------------------------------|--------------------|-----------------------------------|------------------|----------------------------------|-------------------------------|---------------------------------|-----------------------------|
| CH(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH (65a) | 3.29               | 0.7                               | 4.37             | > 5                              | > 5                           | 5.3                             | 0.8                         |
| Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH (65c) | 4.45               | 1.8                               | 4.37             | 1.4                              | 0.17                          | 0.34                            | 0.34                        |
| Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH (65c) | 3.88               | 1.8                               | 4.91             | 0.23                             | 0.04                          | 0.34                            | 0.68                        |
| Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH (65d) | 4.29               | 2.2                               | 4.91             | 2.9                              | 0.18                          | 0.72                            | 1.4                         |
| F(CH <sub>2</sub> ) <sub>11</sub> COOH (66a)                                  | 5.34               | 3.3                               | 4.91             | 1.9                              | 0.12                          | 1.2                             | <0.03                       |
| I(CH <sub>2</sub> ) <sub>11</sub> COOH (66b)                                  | 4.82               | 2.7                               | 4.91             | 2.7                              | 0.09                          | 0.17                            | 0.17                        |
| CH(CH <sub>2</sub> ) <sub>11</sub> COOH (66c)                                 | 5.03               | 2.9                               | 4.91             | 2.2                              | 0.14                          | 0.18                            | <0.04                       |
| Br(CH <sub>2</sub> ) <sub>11</sub> COOH (66d)                                 | 3.88               | 1.8                               | 4.91             | 2.7                              | 0.09                          | 0.3                             | 0.09                        |
| MeO(CH <sub>2</sub> ) <sub>11</sub> COOH (66e)                                | 5.47               | 3.4                               | 4.91             | > 5                              | 0.08                          | 3.1                             | 2.4                         |
| EtS(CH <sub>2</sub> ) <sub>11</sub> COOH (66f)                                | 6.59               | 4.5                               | 4.91             | > 5                              | 0.04                          | 1.1                             | 2.3                         |
| Ph(CH <sub>2</sub> ) <sub>11</sub> COOH (66g)                                 | 4.88               | 2.8                               | 4.91             | 1.3                              | 0.16                          | 0.3                             | 0.3                         |
| N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> COOH (66h)                    | 6.14               | 4.1                               | 4.91             | > 5                              | > 5                           | > 5                             | 5.3                         |
| PhO(CH <sub>2</sub> ) <sub>11</sub> COOH (67a)                                | 3.36               | 1.3                               | 4.91             | > 5                              | 0.36                          | 0.72                            | 1.4                         |
| EtS(CH <sub>2</sub> ) <sub>10</sub> COOH (67b)                                | 4.95               | 2.9                               | 4.91             | 5.1                              | 0.08                          | 0.63                            | 1.3                         |
| Br(CH <sub>2</sub> ) <sub>10</sub> COOH (67c)                                 | 4.51               | 2.4                               | 4.91             | 1.2                              | 0.08                          | 0.08                            | 0.3                         |
| 4-1-PhO(CH <sub>2</sub> ) <sub>10</sub> COOH (67d)                            | 6.89               | 4.8                               | 4.91             | > 5                              | > 5                           | 1.5                             | 3.1                         |
| PhO(CH <sub>2</sub> ) <sub>10</sub> COOH (67e)                                | 5.62               | 3.6                               | 4.91             | > 5                              | 0.14                          | 1.1                             | 2.2                         |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(F)COOH (68a)                            | 6.20               | 2.4                               | 2.83             | > 5                              | 5.1                           | 1.3                             | 5.1                         |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(OH)COOH (68b)                           | 5.17               | 2.1                               | 4.01             | > 5                              | 5.1                           | > 5                             | 2.6                         |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOH (68c)                           | 6.93               | 3.0                               | 3.06             | 0.04                             | 0.02                          | 0.01                            | <0.04                       |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(I)COOH (68d)                            | 7.25               | 3.4                               | 3.30             | 0.1                              | 0.03                          | 0.01                            | 0.1                         |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(Cl)COOH (68e)                           | 6.72               | 2.8                               | 2.99             | 0.59                             | 0.04                          | 0.04                            | 0.59                        |
| Ph(CH <sub>2</sub> ) <sub>9</sub> COOH (69)                                   | 5.56               | 3.5                               | 4.91             | 2.5                              | 0.16                          | 0.16                            | 1.3                         |
| Me(CH <sub>2</sub> ) <sub>12</sub> COOH (70)                                  | 5.97               | 3.9                               | 4.91             | > 5                              | > 5                           | > 5                             | 2.7                         |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOMe (71a)                          | 7.29               | 7.2                               | —                | > 5                              | 1.9                           | > 5                             | 0.49                        |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOEt (71b)                          | 7.81               | 7.7                               | —                | > 5                              | 3.7                           | > 5                             | 0.93                        |
| Me(CH <sub>2</sub> ) <sub>11</sub> CH(Br)COOPr-1 (71c)                        | 8.33               | 8.2                               | —                | > 5                              | > 5                           | > 5                             | > 5                         |
| Amphotericin B                                                                |                    |                                   |                  | 0.0002                           | 0.0004                        | 0.0009                          | 0.003                       |
| Acetone                                                                       |                    |                                   |                  | > 5                              | > 5                           | > 5                             | > 5                         |
| Methanol                                                                      |                    |                                   |                  | > 5                              | > 5                           | > 5                             | > 5                         |
| Fluconazole                                                                   |                    |                                   |                  | 0.007                            | ND <sup>e</sup>               | 0.03                            | ND                          |
| DMSO                                                                          |                    |                                   |                  | 12.5% v/v                        | 6.3% v/v                      | 6.3% v/v                        | 6.3% v/v                    |

<sup>a</sup> The result is the average of three separate experiments. <sup>b</sup> Partition coefficient of the fatty acid calculated using the Prolog<sup>®</sup> 5.1 program. <sup>c</sup> Distribution coefficient of the fatty acid at pH 7.0 calculated using the Prolog<sup>®</sup> prediction program. <sup>d</sup> Calculated dissociation constant. <sup>e</sup> ND = Not determined.



of myristoylation is the major mechanism involved. The observation that myristic acid (70) was devoid of antifungal activity is in agreement with the fact that myristic acid analogs inhibit NMT myristoylation from eliciting their antifungal activity by competing with myristic acid for a binding site(s) on NMT. It was of interest to ascertain whether the activity exhibited by myristoyl analogs in the coupled *in vitro* enzyme assay such as *S. cerevisiae* NMT (Kishore *et al.*, 1993) could accurately forecast antifungal activity. A comprehensive description of the *in vitro* NMT assay, provided in earlier publications, showed that at 0.5 mM ( $\pm$ )-2-bromotetradecanoic acid (68c) reduced NMT activity by 86%. However, ( $\pm$ )-2-fluorotetradecanoic acid (68a), ( $\pm$ )-2-hydroxytetradecanoic acid (68b), and myristic acid (70) provided only 27%, 53% and 27% inhibition, respectively (Paige *et al.*, 1990). This is in agreement with our data showing that ( $\pm$ )-2-bromotetradecanoic acid (68c) is a more potent antifungal agent than ( $\pm$ )-2-fluorotetradecanoic acid (68a) and ( $\pm$ )-2-hydroxytetradecanoic acid (68b).

2-Bromomyristic acid (68c) may exert its antifungal activity by a mechanism similar to 2-bromopalmitic acid. 2-Bromopalmitate and 2-bromopalmitoyl-CoA have been shown to inhibit a variety of enzymes and proteins associated with lipid metabolism. Both compounds were non-competitive inhibitors of microsomal mono- and diacylglycerol acyltransferases that are involved in triacylglycerol biosynthesis. Brominated acyl-compounds might inhibit a variety of membrane-bound effects after insertion into the membrane and formation of adducts with susceptible amino acid residues or with terminal amino groups. The effect of adduct formation on a given enzyme activity would depend on the availability of exposed residues, the function of those residues within the catalytic site, and the localized concentration of the brominated compound relative to that of membrane phospholipids. The brominated acyl-compounds could act as inhibitors, not because they possess a specific affinity for the active site of the microsomal acyltransferases, but because the acyl chains preferentially associate with membranes and, thus, can interact closely with many intrinsic membrane proteins. It is possible that brominated acyl chains insert into



bilayers and thereby have ready access to proteins within the bilayers. Similarly, soluble proteins that bind fatty acids, such as the fatty acid binding proteins, may also be accessible to brominated acyl compounds (Coleman *et al.*, 1992).

NMT displays a high degree of specificity for some fatty acyl-CoA's *in vitro*. There was a poor correlation between activity in the coupled *in vitro* NMT assay system and the antifungal effect for some of the analogs examined. The lack of such a correlation emphasizes the need to compare the substrate specificities of the NMT employed with the corresponding fungal enzyme. This information, together with an assessment of the mechanism of analog uptake into the fungi and the pathways of their subsequent metabolic processing, should allow one to develop systems that will more accurately predict the ability of different classes of myristic acid analogs to inhibit fungi replication (Devadas *et al.*, 1992). The data acquired can be used to determine differences between fungi with respect to the NMT-fatty acyl-CoA interaction. Each type of NMT must have specific binding sites for interaction with inhibitors since some fatty acids such as 67a, 67b and 67e lack antifungal activity against *C. albicans*, but were active against *C. neoformans*.

### 3.3.1.1.2. Antiviral Activity of myristic acid analogs

#### 3.3.1.1.2.1 *In vitro* Anti-HIV Activities of myristic acid analogs

The procedure employed in the United States National Institutes of Health's antiviral assay to evaluate activity against human immunodeficiency virus (HIV) is designed to detect agents acting at any stage of the virus reproductive cycle. Agents that interact with virions, cells, or virus gene-products to interfere with viral activities will protect cells from cytolysis. This assay is suitable to determine the anti-HIV activity of compounds that act by different mechanisms of action. Although this assay does not discriminate between inhibition of virus binding/penetration and inhibition of intracellular replication, it has been reported that myristic acid analogs traverse the cell membrane and act as substrates for NMT. Recent metabolic labeling studies using tritiated oxatetradecanoic acids with an oxygen at C6, C11, and C13, using several cultured mammalian cell lines, indicated that these low molecular weight compounds

readily traverse the cell membrane and are substrates for both acyl CoA synthetase and NMT (Johnson *et al.*, 1990). Myristoyl analogs are selectively incorporated into distinct, yet overlapping subsets of cellular *N*-myristoylproteins. This selective analog-specific incorporation presumably occurs due to cooperative interactions between NMT's acyl-CoA peptide binding sites and the enzyme's ordered reaction mechanism. The antiviral activity of additional myristic acid analogs containing halogen, oxygen, sulfur, azido and aromatic substituents have been investigated to determine the effects of physicochemical properties and their correlation with anti-HIV activity. First, these compounds show a large variation in the physicochemical parameter Log P (3.3-6.9) (Table 3.4). Second, the importance of the steric size of substituents for different aromatic (66g, 66i, 67d, 67e, 69) and halo (66a-d) derivatives could help to identify the putative binding site for the omega terminal of these fatty acids. Finally, the interatomic distance between the CO and terminal heteroatom, which varies in different compounds, can be used to explain relative activity in a series of myristoyl compounds. Studies involving substituent variation will help to confirm that the acyl-CoA binding site may possess a complex sensor that is sensitive to the distance between the carboxyl and the heteroatom moieties of the fatty acid, and the steric volume at the omega terminus as proposed by Kishore *et al.*, (1993).

The results from the *in vitro* anti-HIV screen are presented in Tables 3.4 and 3.5. Since most of the myristoyl analogs investigated exhibited weak anti-HIV activity, the EC<sub>50</sub>, IC<sub>50</sub> and selectivity index could not be determined. Accordingly, inhibition of viral replication (% protection) was scored as a percentage reduction in the virus-induced cytopathic effect (CPE, Table 3.4). Cytotoxicity (IC<sub>50</sub>) values for the test compounds and the test compound concentration required to provide the specified level of protection to infected CD4 lymphocytes are presented in Table 3.4. Some of the important results for individual subclasses of the myristoyl analogs investigated are summarized below.

Table 3.4. Anti-HIV activity and selected physicochemical properties of the myristic acid analogs.

Table 3.4. Anti-HIV activity and selected physicochemical properties of the myristate and arylacetate derivatives.

| No.              | Structure                                                               | Log P <sup>a</sup> | Log D <sub>7.4</sub> <sup>b</sup> | IC <sub>50</sub> (μM) <sup>c</sup> | Concentration (μM) <sup>d</sup>     |      |      |      |       |
|------------------|-------------------------------------------------------------------------|--------------------|-----------------------------------|------------------------------------|-------------------------------------|------|------|------|-------|
|                  |                                                                         |                    |                                   |                                    | 0.6                                 | 6.3  | 20.0 | 63.2 | 200.0 |
|                  |                                                                         |                    |                                   |                                    | Percent Protection (%) <sup>e</sup> |      |      |      |       |
| 65a              | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH | 3.29               | 0.3                               | > 200                              | 5.8                                 | 7.0  | 2.2  | NA   | 39.2  |
| 65c              | Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH | 4.45               | 1.4                               | 83.9                               | NA                                  | NA   | 4.8  | NA   | NA    |
| 65d              | Me(CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> COOH | 3.88               | 1.4                               | 89.1                               | 1.3                                 | 1.4  | 13.4 | 37.3 | 9.2   |
| 65e              | Me(CH <sub>2</sub> ) <sub>8</sub> S(CH <sub>2</sub> ) <sub>2</sub> COOH | 4.95               | 2.5                               | 60.0                               | 4.2                                 | 11.2 | 5.8  | 4.2  | NA    |
| 66a              | F(CH <sub>2</sub> ) <sub>11</sub> COOH                                  | 4.29               | 1.8                               | > 200                              | NA                                  | 0.8  | 10.4 | 48.6 | 21.5  |
| 66b              | I(CH <sub>2</sub> ) <sub>11</sub> COOH                                  | 5.34               | 2.9                               | 110                                | 13.2                                | 29.5 | 45.3 | 70.5 | NA    |
| 66c              | Cl(CH <sub>2</sub> ) <sub>11</sub> COOH                                 | 4.82               | 2.3                               | 92.5                               | NA                                  | NA   | 14.8 | 5.6  | NA    |
| 66d              | Br(CH <sub>2</sub> ) <sub>11</sub> COOH                                 | 5.03               | 2.5                               | 165                                | 14.9                                | 25.9 | 35.2 | 78.6 | 38.4  |
| 66e              | MeO(CH <sub>2</sub> ) <sub>11</sub> COOH                                | 3.88               | 1.4                               | > 200                              | NA <sup>f</sup>                     | NA   | 32.0 | 10.8 | 18.0  |
| 66f              | EtS(CH <sub>2</sub> ) <sub>11</sub> COOH                                | 5.47               | 3.0                               | 38.6                               | 31.7                                | 47.0 | 96.6 | 18.3 | NA    |
| 66g              | Pr(CH <sub>2</sub> ) <sub>11</sub> COOH                                 | 6.59               | 4.1                               | 22.8                               | NA                                  | 6.4  | 15.3 | 6.8  | NA    |
| 66h              | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> COOH                    | 4.88               | 2.4                               | 20.0                               | NA                                  | 12.2 | 26.6 | 7.3  | NA    |
| 66i              | PhO(CH <sub>2</sub> ) <sub>11</sub> COOH                                | 6.14               | 3.7                               | 63.6                               | 1.5                                 | NA   | 18.0 | 1.6  | NA    |
| 66j              | HO(CH <sub>2</sub> ) <sub>11</sub> COOH                                 | 3.26               | 1.4                               | > 200                              | 17.0                                | 31.4 | 24.7 | 28.9 | 26.6  |
| 67a              | MeO(CH <sub>2</sub> ) <sub>10</sub> COOH                                | 3.36               | 0.9                               | > 200                              | NA                                  | NA   | 0.9  | 6.2  | 18.4  |
| 67b              | EtS(CH <sub>2</sub> ) <sub>10</sub> COOH                                | 4.95               | 2.5                               | 66.4                               | NA                                  | 11.2 | 14.9 | 33.2 | NA    |
| 67d              | 4-IPhO(CH <sub>2</sub> ) <sub>10</sub> COOH                             | 6.89               | 4.4                               | 29.9                               | NA                                  | 6.5  | NA   | NA   | NA    |
| 67e              | PhO(CH <sub>2</sub> ) <sub>10</sub> COOH                                | 5.62               | 3.2                               | 41.6                               | NA                                  | 0.8  | 9.4  | 1.6  | NA    |
| 67f              | H <sub>2</sub> N(CH <sub>2</sub> ) <sub>10</sub> COOH                   | 2.81               | -1.0                              | > 200                              | 18.9                                | 6.99 | 2.2  | NA   | 3.1   |
| 68c              | Me(CH <sub>2</sub> ) <sub>11</sub> CH(Ph)COOH                           | 6.93               | 2.8                               | 108                                | —                                   | —    | —    | —    | NA    |
| 69               | Ph(CH <sub>2</sub> ) <sub>9</sub> COOH                                  | 5.56               | 2.5                               | —                                  | —                                   | —    | —    | —    | —     |
| 70               | Me(CH <sub>2</sub> ) <sub>12</sub> COOH                                 | 5.97               | 3.5                               | 100                                | 4.3                                 | 1.0  | 10.9 | 24.3 | —     |
| AZT <sup>g</sup> | —                                                                       | 0.06               | 0.06                              | > 100                              | —                                   | —    | —    | —    | —     |
| 3TC <sup>h</sup> | —                                                                       | -0.27              | -0.57                             | > 100                              | —                                   | —    | —    | —    | —     |
|                  |                                                                         |                    |                                   |                                    | EC <sub>50</sub> = 0.01             |      |      |      |       |
|                  |                                                                         |                    |                                   |                                    | EC <sub>50</sub> = 0.07             |      |      |      |       |

<sup>a</sup> Partition coefficient of the fatty acid calculated using the PrologP 5.1 program (Parang *et al.*, 1996). <sup>b</sup> Distribution coefficient of the fatty acid at pH 7.4 calculated using the PrologD 2.0 prediction program. <sup>c</sup> IC<sub>50</sub> is defined as the test compound concentration required to reduce the number of viable untreated CD4 lymphocytes by 50%. <sup>d</sup> Concentration of the test compound that produces the percentage protection specified against HIV infected T-lymphocytes. <sup>e</sup> The percentage of surviving HIV-infected cells untreated with the test compound (at the concentration indicated) relative to uninfected, treated controls (the value is the average of 2-5 separate experiments). <sup>f</sup> NA = No Activity. <sup>g</sup> Data adopted from NIH. <sup>h</sup> Schinazi *et al.*, (1992a).

The expectation that some dramatic difference would be observed for structurally different myristoyl analogs was not realized, although certain fatty acids show a difference in cytotoxicity ( $IC_{50}$ ) against CD4 uninfected lymphocytes. Of these, 12-iodododecanoic acid **66b**, 12-bromododecanoic acid **66d** and 12-thioethyldodecanoic acid **66f**, were moderately active against CD4 HIV-infected lymphocytes. For compounds having moderate activity (**66b**, **66d** and **66f**), the concentration that produced a 50% cytotoxic effect on uninfected cells was at least 3-4 fold larger than its corresponding  $EC_{50}$  value (Table 3.5). At higher concentrations (e.g. 63.2  $\mu M$ ), a decreased activity for **66f** was observed, probably due to its cytotoxicity ( $IC_{50} = 38.6 \mu M$ ). Another possibility for the unusual decreased activity of some analogs such as **66f**, **66j** at higher concentrations could be due to precipitation of these compounds in the culture medium. (Table 3.4).

#### 3.3.1.1.2.1.1. Thioethers

The most active of the 20 test compounds evaluated in the acute viral propagation assay was 12-thioethyldodecanoic acid **66f**, which produced a 97% reduction in the cytopathic effect of HIV at a 20  $\mu M$  concentration (Table 3.4). The  $EC_{50}$  for 12-thioethyldodecanoic acid **66f** was 9.37  $\mu M$ , and its  $IC_{50}$  was 38.6  $\mu M$ . This latter compound **66f** is therefore moderately active when compared to the active reference nucleosides AZT and 3TC (Tables 3.4 and 3.5).

#### 3.3.1.1.2.1.2. 12-Halo-substituted fatty acids

12-Halododecanoic acids such as the 12-iodo- **66b** and 12-bromo- **66d** analogs were only weakly cytotoxic, while simultaneously increasing the percentage of surviving HIV-infected cells (70-79%) at a test compound concentration of 63-70  $\mu M$ . Several test compounds such as 12-iodododecanoic acid **66b**, 12-bromododecanoic acid **66d** and 12-fluorododecanoic acid **66a** produced 48-79% reduction in virus-induced cytopathicity at a concentration that was 2 to 4-fold lower than the test drug  $IC_{50}$ .

Table 3.5. Comparative potency and selectivity for three fatty acids as inhibitors of HIV replication in CD4 cells (CEM-SS cell line).

| Compound         | IC <sub>50</sub> , $\mu\text{M}^a$ | EC <sub>50</sub> , $\mu\text{M}^b$ | TI <sub>50</sub> <sup>c</sup> |
|------------------|------------------------------------|------------------------------------|-------------------------------|
| 66b              | 110 $\pm$ 0                        | 30 $\pm$ 8.9                       | 4.5 $\pm$ 2.9                 |
| 66d              | 165 $\pm$ 20.6                     | 38 $\pm$ 10.7                      | 4.5 $\pm$ 1.0                 |
| 66f              | 38.6 $\pm$ 14.1                    | 9.37 $\pm$ 7.1                     | 3.0 $\pm$ 0.9                 |
| AZT              | 100                                | 0.01                               | 10000                         |
| 3TC <sup>d</sup> | >100                               | 0.07                               | >1429                         |

<sup>a</sup> IC<sub>50</sub> is defined as the inhibitory concentration, required to reduce the number of viable untreated CD4 lymphocytes by 50% (mean  $\pm$  SD, n = 5). <sup>b</sup> EC<sub>50</sub> is defined as the 50% antiviral effective concentration, required to produce a 50% reduction in the cytopathic HIV effect in CD4 lymphocytes (mean  $\pm$  SD, n = 5). <sup>c</sup> TI<sub>50</sub> is defined as the therapeutic index (IC<sub>50</sub> / EC<sub>50</sub>) (mean  $\pm$  SD, n = 5). <sup>d</sup> Schinazi *et al.*, 1992a (CEM cell line).

### 3.3.1.1.2.1.3. Phenylsubstituted fatty acids

To assess whether antiviral activity and cytotoxicity are affected by an aromatic ring or a heteroatom, a number of aromatic myristic acid analogs were synthesized. The *in vitro* data for a series of aromatic myristic acid analogs show that 12-phenyldodecanoic acid 66g is more cytotoxic than 10-phenyldecanoic acid 69 toward uninfected CD4 lymphocytes. 12-Phenoxydodecanoic acid 66i, 11-phenoxyundecanoic acid 67e and 11-(4-iodophenoxy)undecanoic acid 67d were synthesized to determine the effect of oxygen substitution in conjunction with aromatic rings. These aromatic ether analogs (66i, 67e and 67d) were generally less active than the corresponding aromatic analogs (66g and 69) and the alkyl ether analog 66e against HIV-infected CD4 lymphocytes cells.

It was found that the anti-HIV activity of myristic acid analogs was not affected by oxygen substitution or placement of an iodine substituent at the *para*-position of a phenyl ring. All of these analogs exhibited very weak activity, without a change in cytotoxicity relative to the corresponding aromatic or ether analog, against HIV-



infected CD4 lymphocyte cells. Kishore *et al.*, (1991) proposed that compounds having a terminal phenyl should not fit into the postulated conical receptor, and therefore should exhibit a lower activity than analogs having terminal methyl groups. Our *in vitro* anti-HIV data for the aromatic fatty acids (66g, 66i, 67d, 67e, 69) confirm this postulation.

#### 3.3.1.1.2.1.4. Other myristoyl analogs

A number of compounds (66e, 67a, 65a, 66a, 66j, 67f) exhibited low cytotoxicity ( $IC_{50} > 200 \mu M$ , see Table 3.4), but several compounds, including 12-thioethyldodecanoic acid 66f, 12-phenyldodecanoic acid 66g, 11-phenoxyundecanoic acid 67e and 11-(4-iodophenoxy)undecanoic acid 67d were substantially more cytotoxic ( $IC_{50} = 23-42 \mu M$ ). These data showed that all of the sulfur containing compounds were more cytotoxic than their oxygen substituted analogs (12-thioethyldodecanoic acid 66f,  $IC_{50}$  of  $38.6 \mu M$ ; 12-methoxydodecanoic acid 66e,  $IC_{50} > 200 \mu M$ ). The combination of oxygen at the 4-position and chlorine at the 12-position in 12-chloro-4-oxadodecanoic acid 65a resulted in a lower cytotoxicity than observed for either 4-oxatetradecanoic acid 65c or 12-chlorododecanoic acid 66c against HIV infected T cells. The myristoyl analogs investigated were, at most, moderately effective with respect to their ability to protect HIV infected CD4 lymphocytes.

A comparison of the anti-HIV activity for myristoyl analogs investigated in this study, excluding compounds 66b, 66d and 66f, with previously reported data warrants discussion. Recent studies have shown that some oxatetradecanoic acid analogs such as 12-methoxydodecanoic acid 66e inhibit replication of HIV-I infected cells without accompanying cellular toxicity in both acutely and chronically infected CD4 H9 cells (Devadas *et al.*, 1992). Metabolic labeling studies using tritiated 12-methoxydodecanoic acid 66e revealed that it was selectively incorporated into subsets of cellular *N*-myristoylproteins. Compound 66e was metabolized slowly, and only a small fraction of the drug entered the cell (Bryant *et al.*, 1993). Compound 66e was potent as judged by its ability to produce a concentration-dependent decrease in reverse transcriptase activity,

p24 antigen levels, and syncytia formation. For example, an 80-90% reduction in reverse transcriptase activity was achieved when a 20  $\mu$ M test compound solution was added to serum-containing medium (Bryant *et al.*, 1991, 1993). In contrast, our data indicate that compound 66e produced only a 32% reduction in virus replication (infected CD4 lymphocyte in a CEM-SS cell line) at a 20  $\mu$ M test compound concentration. This result is in agreement with *in vitro* NMT assay data which showed that 66e is a poor NMT substrate (Kishore *et al.*, 1991). 4-Oxatetradecanoic acid 65c inhibits HIV-1 assembly (Kishore *et al.*, 1993). It has been reported that 65c produced a 50% reduction of HIV-1 replication in acutely infected human T-lymphocyte cell lines at a concentration of 18  $\mu$ M (Langner *et al.*, 1992). 4-Oxatetradecanoic acid (65c) is able to inhibit replication of HIV-1 in acutely infected T-lymphocytes. The dose of compound that produces a 50% reduction in the cytopathic effect of the virus (18  $\mu$ M) is 4-fold lower than its IC<sub>50</sub> (80  $\mu$ M) (Bryant *et al.*, 1993). In contrast to reported data, in this study a 20  $\mu$ M concentration of 65c produced only a 4.8% inhibition of the CPE. It has been reported that 12-azidododecanoic acid 66h, like 12-methoxydodecanoic acid 65c, inhibits HIV-1 production by 60-70% in chronically infected T-lymphocyte cell lines at a concentration of 10-50  $\mu$ M without appreciable associated cellular toxicity (Devadas *et al.*, 1992). In spite of the previously reported data, the maximum protection observed in our *in vitro* assay against CD4 HIV infected lymphocytes at a 20  $\mu$ M concentration of 66h was 26%. Results obtained in this study for 66e, 65c, and 66h do not indicate a similar high anti-HIV activity for these analogs. The differences in anti-HIV activity observed in this study, compared to literature data, could be due to fact that 100% dimethyl sulfoxide (DMSO) was used as solvent to dissolve the test compound rather than ethanol. This explanation is based on a report by Harper *et al.*, (1996) that stock solutions of 2-hydroxymyristic acid prepared in DMSO, rather than ethanol, did not significantly inhibit VZV or HIV-1 replication, suggesting that the use of DMSO as a 'universal solvent' for drug screening may result in false negative results for some compounds. Furthermore, the NIH method which measures the *in vitro* cytopathic effect of HIV-1 on suitable host cells, is different from other anti-HIV assays that are used to analyze viral antigen P24 synthesis and reverse

transcriptase (RT) activity.

### 3.3.1.1.2.2. *In vitro* anti-HBV activities of myristic acid analogs

Three fatty acids (66e, 66f, and 68c) were evaluated against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2). The EC<sub>50</sub> for selected compounds 66e, 66f, 68c, 2', 3'-dideoxycytidine (ddC) and 2',3'-dideoxy-3'-thiacytidine (3TC) (Xie *et al.*, 1995) [compound number (EC<sub>50</sub> for intracellular DNA)] in HBV 2.2.15 (HepG2 cell line) for 14 days was: 66e (81.8  $\mu$ M), 66f (17.8  $\mu$ M), 68c (62.2  $\mu$ M), ddC (2.8  $\mu$ M) and 3TC (0.076  $\mu$ M) where each datum represents the mean of duplicate samples. Among the fatty acids, 12-thioethyldodecanoic acid 66f showed the most potent activity. No cytotoxicity values were determined in this assay, since this method is used solely as an *in vitro* model for screening anti-hepatitis virus therapeutics. Intracellular DNA was assayed by dot hybridization and phosphodensitometry. Compound 66e was selected for further studies to gain a better understanding regarding its efficacy and toxicity since compound 66e has been reported previously to exhibit medium anti-HIV activity (Bryant *et al.*, 1991, 1993).

12-Methoxydodecanoic acid 66e, which was also evaluated against chronically HBV-producing human liver cells (2.2.15), exhibited medium activity against HBV DNA replication in 2.2.15 cells (HBV virion extracellular DNA, HBV RI intracellular DNA) and it was less toxic than dideoxydidehydrocytidine (ddC) (see Table 3.6). This latter assay, which serves as a model for chronic HBV infection in man, provided additional information regarding the activity and toxicity for compound 66e.

12-Methoxydodecanoic acid 66e was moderately active as an inhibitor of HBV DNA replication in 2.2.15 cell culture (HBV RI intracellular DNA, HBV virion extracellular DNA) compared to the reference compound ddC and is less toxic. The EC<sub>50</sub> values for compound 66e in these two assays are different, since the harvesting time, the duration of exposure, and the time at which cells were exposed to the test compound, differ.

Table 3.6. Relative *in vitro* potency and selectivity of 12-methoxydodecanoic acid (66e), 3TC, and ddC as inhibitors of HBV DNA replication in 2.2.15 cell culture (HBV RI intracellular DNA, HBV virion extracellular DNA).

| No.              | IC <sub>50</sub><br>( $\mu$ M) <sup>a</sup> | EC <sub>50</sub> ( $\mu$ M) <sup>b</sup> |     | EC <sub>90</sub> ( $\mu$ M) <sup>c</sup> |     | EC <sub>50</sub> ( $\mu$ M) <sup>b</sup> |     | EC <sub>90</sub> ( $\mu$ M) <sup>c</sup> |     | SI <sup>d</sup> |               |
|------------------|---------------------------------------------|------------------------------------------|-----|------------------------------------------|-----|------------------------------------------|-----|------------------------------------------|-----|-----------------|---------------|
|                  |                                             | Extracellular                            | DNA | Extracellular                            | DNA | Intracellular                            | DNA | Intracellular                            | DNA | Extracellular   | Intracellular |
| 66e              | 378                                         | 22 $\pm$ 1.8                             |     | 60 $\pm$ 6.3                             |     | 31 $\pm$ 3.4                             |     | 90 $\pm$ 11                              |     | 6.3             | 4.2           |
| ddC <sup>e</sup> | 252 $\pm$ 28                                | 1.3 $\pm$ 0.1                            |     | 6.8 $\pm$ 0.7                            |     | 2.6 $\pm$ 0.3                            |     | 13 $\pm$ 1.8                             |     | 37              | 19            |
| 3TC <sup>f</sup> | 1180                                        | 0.033 $\pm$ 0.004                        |     | 0.188 $\pm$ 0.020                        |     | 0.076 $\pm$ 0.008                        |     | 0.293 $\pm$ 0.031                        |     | 6280            | 4030          |

<sup>a</sup> IC<sub>50</sub> = 50% cytotoxic concentration. <sup>b</sup> EC<sub>50</sub> = 50% effective concentration. <sup>c</sup> EC<sub>90</sub> = 90% effective concentration. <sup>d</sup> Selectivity Index (S.I.: IC<sub>50</sub>/EC<sub>90</sub>). <sup>e</sup> ddC = 2', 3'-dideoxycytidine. <sup>f</sup> 2', 3'-dideoxy-3'-thiacytidine. (Xie *et al.*, 1995).

Additional studies are required to make definitive conclusions regarding the anti-HBV effects exhibited by these myristoyl compounds. 12-Thioethyldodecanoic acid **66f** showed the highest anti-HBV activity, compared to other fatty acids at a lower concentration (5  $\mu$ M) against HBV producing 2.2.15 cell cultures derived from Hep G2. Although the mechanism(s) by which these myristoyl analogs inhibit DNA synthesis to produce their anti-HBV effect is unclear, it may be due at least in part to drug cytotoxicity on DNA. Infected cells are also more sensitive to inhibitors than uninfected cells. HBV replication is cell cycle dependent which supports the concept of effect on viral DNA replication by these agents (Ozer *et al.*, 1996).

#### 3.3.1.1.2.3. *In vitro* anti-DHBV activities of myristic acid analogs

A major advance in the ability to access new approaches to therapy of hepatitis B has been the identification of animal models of this infection like duck hepatitis B virus (DHBV). Duck hepatitis B virus (DHBV), a member of the family *Hepadnaviridae*, has a similar virion structure and genome organization to that of human HBV. Thus, DHBV has been used extensively to screen potential drugs to control chronic HBV infection (Lin *et al.*, 1996). A group of myristic acid analogs was also evaluated using a duck hepatocyte culture system infected with DHBV. At a test compound concentration of 10  $\mu$ g/mL, none of fatty acids significantly inhibited the replication of DHBV-infected hepatocytes. Viral replication (the values are the average of two different experiments) was estimated from the amount of DHBV DNA present in the supernatant of the cell culture after incubation with the test compound (10  $\mu$ g/mL). The data are expressed as a percentage of control values where the cell culture was not incubated with the test compound. The % of viral replication for selected compounds [compound number (% of viral replication)] was **65c** (49.1%); **65d** (94.8%); **66b** (68.9%); **66c** (78.9%); **66d** (80.2%); **66e** (64.4%); **66f** (62.1%); **67b** (65.1%); **68a** (77.2%); **68c** (93.0%); **68d** (89.2%); **68e** (53.0%); **70** (78.9%); and control (100.0%). The most potent compound in this group, 4-oxatetradecanoic acid **65c**, inhibited DHBV-infected hepatocytes by 49%. These results clearly illustrate that evaluation of the anti-DHBV activity in one cell culture may not be



representative of the antiviral activity of the drug. The reason for the observed differences between the different cell systems may be related to metabolic peculiarities, as documented for HepG2 2.2.15 cells (Aoki-sei *et al.*, 1991). Also the genomic organization of the hepadnavirus DNA seems to differ from one cell system to another: no integration is observed for DHBV, whereas in HepG2 2.2.15 cells HBV DNA shows two integration bands. It is unknown whether this differential genomic organization influences the susceptibility of viral DNA synthesis to the antiviral drugs. Part of the discrepancies noted between the results in different cell lines may also be ascribed to the experimental design (time, duration of drug administration) and the choice of the drug concentrations (Heijntink *et al.*, 1993).

Macrae *et al.*, (1991) reported that myristoylation of the duck hepatitis B virus (DHBV) envelope is essential for infectivity, but not for virus assembly. Myristoylation is most likely required as an early step in the life cycle involving the entry of uncoated virus particles. HBV particle formation can occur in the presence or absence of the myristoyl moiety. Even though myristoylation-null HBV mutants form virion particles, these particles are noninfectious (Macrae *et al.*, 1991). In this study, compounds were tested only to detect a significant effect on DNA synthesis, but not virus infectivity. Therefore, these data cannot be used to define a relationship between myristylation and anti-(D)HBV activity.

The myristic acid analogs 66e, 66f and 68c exhibit differential activity between DHBV and HBV. It is not known whether this differential genomic organization influences the susceptibility of viral DNA synthesis upon exposure to antiviral drugs (Heijntink *et al.*, 1993). HBV and DHBV, which belong to the hepadnavirus family, possess different *N*-terminal sequences for myristoylation. Marked differences have also been observed for intracellular metabolism of different compounds in DHBV hepatocytes, and a human HIV cell line, that can effect its antiviral activity. The *N*-terminal sequence for *N*-myristoylated proteins for HBV is GGTSSLPA (Valenzuela *et al.*, 1979), while that for DHBV is GQHPALSM (Macrae *et al.*, 1991). Although myristoylation occurs at the terminal-amino glycine residue, other amino acids are also important in this pathway. More importantly, since these assays measure DNA synthesis, the nucleotide sequence for the hepatitis B

virus genome differs from that for the nucleotide sequence for DHBV. Consequently, DNA synthesis inhibition for these two viruses may be different for the same agent. Another explanation why DHBV production was not significantly affected, may be due to the fact that inhibition was determined in non-growing primary hepatocyte cultures, whereas inhibition of HBV was evaluated in cultures under conditions where cell division is expected.

### **3.3.1.2. Physicochemical properties of myristic acid analogs**

#### **3.3.1.2.1. Retention time and $R_f$ values**

HPLC retention times and  $R_f$  values are shown in Table 3.7.

#### **3.3.1.2.2. Interatomic distance between the CO and the omega heteroatom**

The calculated interatomic distances between the CO and the omega heteroatom were: 15.00 Å (65a), 9.84 Å (65d), 10.15 Å (65e), 14.88 Å (66a), 15.47 Å (66b), 15.18 Å (66c), 15.34 Å (66d), 15.16 Å (66f), 14.86 Å (66i), 14.90 Å (66j), 13.61 Å (67a), 19.42 Å (67d), 13.60 Å (67e) and 13.75 Å (67f).

Table 3.7.  $R_f$  and retention time (mean  $\pm$  SD) values for myristic acid analogs.

| No. | Structure                   | Retention Time (min) <sup>a</sup> | $R_f^b$         | No. | Structure                   | Retention Time (min) | $R_f$              |
|-----|-----------------------------|-----------------------------------|-----------------|-----|-----------------------------|----------------------|--------------------|
| 65a | $Cl(CH_2)_9O(CH_2)_2COOH$   | 6.29 $\pm$ 0.03                   | —               | 67b | $EtS(CH_2)_{10}COOH$        | 4.74 $\pm$ 0.03      | 0.73 $\pm$ 0.07    |
| 65c | $CH_3(CH_2)_9O(CH_2)_2COOH$ | 5.00 $\pm$ 0.11                   | 0.74 $\pm$ 0.07 | 67c | $Br(CH_2)_{10}COOH$         | 4.48 $\pm$ 0.02      | 0.66 $\pm$ 0.07    |
| 65d | $CH_3(CH_2)_4O(CH_2)_7COOH$ | 4.76 $\pm$ 0.02                   | 0.81 $\pm$ 0.04 | 67d | 4- $PhO(CH_2)_{10}COOH$     | 5.33 $\pm$ 0.02      | 0.70 $\pm$ 0.02    |
| 66a | $F(CH_2)_{11}COOH$          | 7.03 $\pm$ 0.15                   | 0.63 $\pm$ 0.04 | 67e | $PhO(CH_2)_{10}COOH$        | 4.66 $\pm$ 0.04      | 0.72 $\pm$ 0.05    |
| 66b | $I(CH_2)_{11}COOH$          | 5.15 $\pm$ 0.05                   | 0.81 $\pm$ 0.07 | 67f | $NH_2(CH_2)_{10}COOH$       | 3.59 $\pm$ 0.03      | 0.0187 $\pm$ 0.003 |
| 66c | $Cl(CH_2)_{11}COOH$         | 4.53 $\pm$ 0.06                   | 0.73 $\pm$ 0.07 | 68a | $CH_3(CH_2)_{11}CH(F)COOH$  | 5.87 $\pm$ 0.04      | 0.29 $\pm$ 0.01    |
| 66d | $Br(CH_2)_{11}COOH$         | 4.79 $\pm$ 0.03                   | 0.74 $\pm$ 0.06 | 68b | $CH_3(CH_2)_{11}CH(OH)COOH$ | 7.38 $\pm$ 0.04      | 0.03 $\pm$ 0.01    |
| 66e | $MeO(CH_2)_{11}COOH$        | 4.64 $\pm$ 0.02                   | 0.77 $\pm$ 0.07 | 68c | $CH_3(CH_2)_{11}CH(Br)COOH$ | 6.21 $\pm$ 0.08      | 0.72 $\pm$ 0.08    |
| 66f | $EtS(CH_2)_{11}COOH$        | 4.94 $\pm$ 0.08                   | 0.82 $\pm$ 0.07 | 68d | $CH_3(CH_2)_{11}CH(N)COOH$  | 6.74 $\pm$ 0.03      | 0.58 $\pm$ 0.05    |
| 66g | $Ph(CH_2)_{11}COOH$         | 5.41 $\pm$ 0.03                   | 0.81 $\pm$ 0.05 | 68e | $CH_3(CH_2)_{11}CH(Cl)COOH$ | 6.61 $\pm$ 0.05      | 0.57 $\pm$ 0.10    |
| 66h | $N_3(CH_2)_{11}COOH$        | 4.54 $\pm$ 0.04                   | 0.34 $\pm$ 0.02 | 69  | $Ph(CH_2)_9COOH$            | 4.73 $\pm$ 0.05      | 0.79 $\pm$ 0.04    |
| 66i | $PhO(CH_2)_{11}COOH$        | 4.74 $\pm$ 0.02                   | 0.78 $\pm$ 0.04 | 70  | $CH_3(CH_2)_{12}COOH$       | 6.36 $\pm$ 0.09      | 0.77 $\pm$ 0.06    |

<sup>a</sup> HPLC retention time using an evaporative light scattering detector, Conditions: acetonitrile/water: 90/10 v/v, flow rate = 1 mL/min, drift tube temperature = 102  $\pm$  0.2  $^{\circ}C$ , gas flow = 2.15 SLPM, solvent press = 7.2-7.4 PSIG. <sup>b</sup>  $R_f$  values (mean  $\pm$  SD) (mobile phase: diethyl ether/hexane, 50 mL, 90:10 v/v); K6F Silica Gel 60  $\text{\AA}$ , Size: 10  $\times$  20 cm Layer thickness: 250  $\mu$  (Whatman) developed by bromocresol green (40 mg/100 mL ethanol, has transition interval that is yellow at pH 3.8 and blue at pH 5.4) or primuline (acetone:water 4:1, 0.01% w/v,  $\lambda_{max}$  = 356 nm).

### 3.3.1.2.2.1. NMT enzyme structure and its relationship with anti-HIV activity of myristic acid analogs

Analysis of over 300 fatty acid analogs suggests that the acyl chain of bound myristoyl-CoA assumes a bent conformation (Kishore *et al.*, 1991, Devadas *et al.*, 1993). (see Figure 3.1). The key features of the model are:

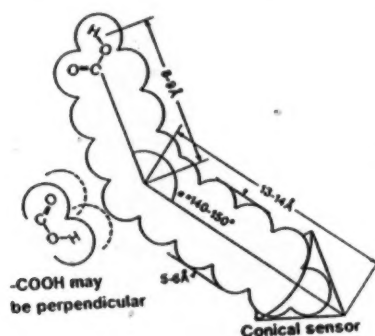


**Figure 3.1.** Hypothetical schematic representation of the conformation of the acyl chain of myristoyl-CoA in the acyl-CoA binding site of *S. cerevisiae* NMT (Kishore *et al.*, 1991).

- (1) The first four or five carbons of the fatty acid chain exist in an extended conformation.
- (2) The  $\omega$  end of the fatty acid (FA) contacts a conical sensing device that detects both length and bulk. The end site appears to possess a complex measuring device that can sense distance along the chain from the carboxyl to the  $\omega$  end of the fatty acid as well as steric volume at the  $\omega$  terminus. The overall shape of this sensor may be conical with the cone and the terminal carbons coaxial. In this proposed model, the relative sensitivity to chain length *versus* steric volume would be determined by the acuteness of the cone (Kishore *et al.*, 1991, Rudnick *et al.*, 1992, Peseckis *et al.*, 1993). For example, 10-phenyldecanoylCoA is an equally good substrate to myristoylCoA; extending the methylene chain by one carbon (11-phenylundecanoylCoA) reduces activity by an order of magnitude (Kishore *et al.*, 1991, Johnson *et al.*, 1993, Rudnick *et al.*, 1993). It has been suggested that the acyl-CoA binding site can detect the distance from the carboxyl to the  $\omega$  terminus of a bound fatty acid.

(3) The acyl chain is bound in a bent conformation. The distance between C1 and a bend located near C5 is a critical determinant for optimal positioning of the acyl-CoA guest in the binding site of the NMT host, and the length of the acyl chain between the bend and the  $\omega$  terminus has an important effect on the stability of the host-guest interaction and/or positioning of the guest in the acyl-CoA binding site.

The interatomic distances between the CO and the omega moieties for compounds 66b, 66d and 66f, all having an omega-heteroatom group, can be used to explain the unpredicted anti-HIV activity exhibited by these compounds. For moderately active compounds (66b, 66d and 66f), the distances between the CO and the heteroatom at the terminal end (sulfur, iodine and bromine) of the molecule are 15.47 Å, 15.34 Å and 15.16 Å, respectively. In the case of other myristoyl analogs, this distance is either shorter or longer (See section 3.3.1.2.2). Changing the halogen to chlorine (66c) from iodine (66b) and bromine (66d) resulted in a significant decrease in antiviral activity. In the case of other 12-halo analogs such as 12-chloro- (66c) and 12-fluoro- (66a), this distance is 15.18 Å and 14.88 Å, respectively. This interatomic distance is similar to that for 66b and 66d in the case of 66c. However, the larger steric volume for bromine, iodine and sulfur, relative to chlorine could be an important factor with respect to binding of the fatty acid analog to its binding site on the NMT enzyme. In addition, the lower partition coefficient for 66c may decrease uptake of the chloro analog.

From these data it may be suggested that specific amino acid residues present in NMT bind to specific sections of the fatty acid backbone, that fatty acid acyl CoA may be rigidly bound by NMT, and that the acyl-CoA binding site is sensitive to the distance between the carboxyl and the omega end of the fatty acid, and to the steric volume at the omega terminus.

### 3.3.1.2.3. Partition coefficient (Log P), distribution coefficient (Log D), and $pK_a$

These theoretical physicochemical parameters were calculated to ascertain whether they correlate with and/ or are determinants of antifungal and anti-HIV activity. It has been known for a long time that as one increases the partition coefficient beyond an optimal value that biological activity falls off eventually becoming inactive since highly



lipophilic compounds no longer circulate in the blood stream but bind to the lipid membranes. Thus, there is often an optimum partition coefficient for a biologically active class of compounds (Hansch *et al.*, 1964). This being true, one cannot expect a linear relation between drug response and Log P to prevail over a large range of P values. The higher the Log P, the tighter the binding between the drug and the first protein or lipid phase with which the drug comes into contact. That is, as P approaches infinity, binding of the drug becomes so firm that such a drug cannot readily cross aqueous phases and tends to localize in the first lipophilic phase with which it comes in contact. As P approaches zero, the drug becomes so water-soluble that it tends to localize in the first aqueous phase into which it enters. Somewhere between zero and infinity there will be a value of P such that the drug will be least hindered in its random journey through macromolecules to its site of action (Hansch *et al.*, 1971).

Log P values were calculated using the PrologP 5.1 program as mentioned in section 4.8.1. To confirm the validity of the Log P calculation, the experimental data were compared with experimental acquired data for some fatty acids. For example, the experimental value of 6.1 (D'Amboise *et al.*, 1982) reported for myristic acid (70) is in good agreement with the calculated Log P value of 5.97.

Ionization of a basic or acidic drug provides an additional factor to consider, since partitioning becomes pH dependent. The distribution coefficient (D) considers the partitioning of all ionic species of a compound. Experimentally this may be measured with a buffered aqueous phase, usually at pH 7.4, the pH of many biological fluids, e.g. plasma. A value obtained in this way is frequently referred to as a distribution coefficient, Log D, rather than Log P value and the two can be related by *pKa* using the following formula (Dunn *et al.*, 1987):

$$\text{Log D} = \text{Log P} + \log (1 + 10^{pKa - pH})$$

In order to calculate the Log D<sub>7.0</sub> value for a test compound, both the logarithms of the partition coefficient (Log P) and the dissociation constant(s) (*pKa*) are needed. To perform these calculations, the test compound structure is drawn graphically, after which the computational program PrologD automatically calculates these parameters by activating the PrologP and PrologD modules of PALLAS.

Calculation of lipophilicities reduces the need for routine and tedious measurement of partition coefficients. If one member of a related series of compounds has a measured lipophilicity, then that of the others may be calculated using fragmental values. This method uses numerical addition or subtraction values for each functionality introduced or deleted, although correction factors should be introduced if the altered functional groups are in close proximity. In principle, calculations may be extended to any structure without the need for measured values. However these calculations do not account for the overall three-dimensional shape of a molecule. Shape may have a large effect on lipophilicity since polar/lipophilic groups can be buried within the molecule distant from solvent (Dunn *et al.*, 1987).

Partition ( $\text{Log } P = 3.3\text{--}7.3$ ) and distribution ( $\text{Log } D_{7.4} = -1.0$  to  $4.4$ ;  $\text{Log } D_{7.0} = 0.7\text{--}4.8$ ) coefficients, which were calculated for the fatty acids investigated (Tables 3.3), extended over a broad range (Parang *et al.*, 1996). It was found that the calculated  $pK_a$  values for most myristic acid analogs investigated was 4.91, except for the 2-substituted and the 4-oxa-analogs. For example, ( $\pm$ )-2-bromotetradecanoic acid (68c) had a calculated  $pK_a$  of 3.06, whereas 4-oxatetradecanoic acid (65c) and 12-chloro-4-oxadodecanoic acid (65a) had a calculated  $pK_a$  value of 4.37.

#### 3.3.1.2.3.1. Correlation of partition coefficient and anti-fungal activity for myristic acid analogs

( $\pm$ )-2-Bromotetradecanoic acid (68c), ( $\pm$ )-2-iodotetradecanoic acid (68d) and ( $\pm$ )-2-chlorotetradecanoic acid (68e) have calculated  $\text{Log } P$  values between 6.7-7.3 ( $\text{Log } D_{7.0}$  between 2.8-3.4) (Table 3.3). There appears to be a relationship between antifungal activity against all fungi tested and the calculated  $\text{Log } P$  for these 2-halo myristic acid analogs where the more lipophilic 2-Br and 2-I analogs are more potent than the less lipophilic 2-F and 2-OH derivatives. These data suggest that there is an optimal partition coefficient range for maximum antifungal activity. Based on previous *in vitro* NMT assay data, the observation that the activities of NMT acyl-CoA substrates diminish with increasing polarity (Devadas *et al.*, 1992), is in agreement with our data for 2-substituted fatty acids.

### 3.3.1.2.3.2. Correlation of partition coefficient and anti-HIV activity for myristic acid analogs

12-Thioethyldodecanoic acid 66f, 12-iodododecanoic acid 66b, and 12-bromododecanoic acid 66d had calculated partition coefficients (Log P) between 5.0-5.5 (Log  $D_{7.4}$  between 2.5-3.0) (Figure 3.2). There does appear to be a direct correlation between anti-HIV activity and Log P for these fatty acids, although the anti-HIV data suggest that the partition coefficient should be in the Log P 5.0-5.5 range for optimal anti-HIV activity, since myristoyl analogs with higher or lower partition coefficients exhibit reduced activity (Parang *et al.*, 1996). Previous *in vitro* NMT assay data showed that the activities of NMT acyl-CoA substrates diminish with increasing polarity (Devadas *et al.*, 1992), which is in agreement with our data, since myristoyl analogs with Log P < 5.0 exhibit weak anti-HIV activities (Figure 3.2). The study of ether-, ketocarbonyl, ester, ester-, and amide- containing analogs indicated that as the polarity (hydrophilicity) of analog-CoAs increases, their activities as NMT substrates decrease (Bryant *et al.*, 1993, Devadas *et al.*, 1993). This relationship is also apparent from studies of compounds containing  $\omega$ -terminal azido, imidazole, triazole and tetrazole, carboxyl, nitro, cyano, and halogen groups (Vincent *et al.*, 1993, Devadas *et al.*, 1995).

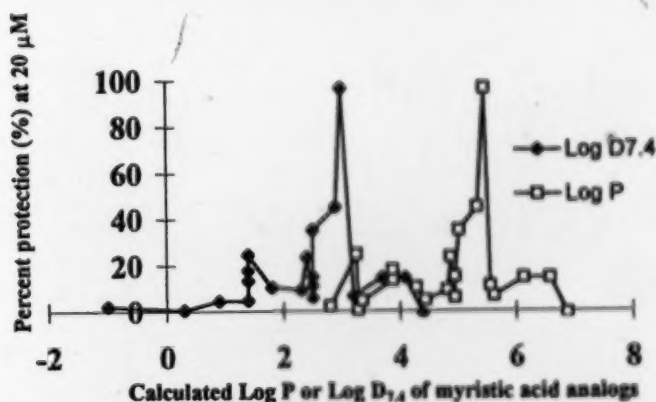


Figure 3.2. Percent protection provided by myristic acid analogs against HIV infected CD4 lymphocytes at 20  $\mu$ M vs calculated Log P or Log  $D_{7.4}$ .

### 3.3.1.2.3.3. Correlation of $pK_a$ with anti-HIV and antifungal activities for myristic acid analogs

The  $pKalc$  3.1 program module was used to calculate  $pK_a$  values, and the validity of these data was established by comparing the experimental values of a few fatty acids with their calculated  $pK_a$  value. For example, the experimental  $pK_a$  value of 5.0 for myristic acid (70) (Drummond *et al.*, 1989) is comparable with the calculated  $pK_a$  value of 4.9. As anticipated, the calculated  $pK_a$  value for 12-chloro-4-oxadodecanoic acid (65a) and 4-oxatetradecanoic acid (65c) was lower (4.37) than that of myristic acid (70) (4.91) due to the electronegative effect of the 4-oxa moiety.

Since a large difference in the  $pK_a$  values of 2-substituted fatty acids is often observed, a more precise calculation was required for these analogs (68a-e). The  $pK_a$  values of the 2-substituted myristic acid analogs (68a-e) listed in Table 3.3 were calculated by subtracting the substituent  $pK_a$  value ( $\Delta pK_a$ ) for a substituent attached to the  $\alpha$ -carbon of an aliphatic acid (Perrin *et al.*, 1981) from the  $pK_a$  value for myristic acid (70,  $pK_a$  = 4.91). The validity of this method was confirmed by comparing experimental and calculated  $pK_a$  values for 2-halo- and 2-hydroxy- derivatives of acetic acid (Rapport *et al.*, 1967) where the magnitude of the  $pK_a$  value correlates with the electronegativity of C-2 substituent of the acid.

No relationship between  $pK_a$  and anti-HIV activity was evident for these myristoyl analogs, since their  $pK_a$  values were generally similar (4.9). This result could be due to the small inductive effect of an alkyl group, an effect that decreases rapidly with increasing distance from the carboxylate substituent. Thus, only atoms which are adjacent to the acidic center will exert a significant effect on the observed  $pK_a$  value (Perrin *et al.*, 1981). However, active antifungal analogs 68c-e had  $pK_a$  values in the range of 2.99-3.30 as mentioned previously (see section 3.3.1.1.4.2).

### 3.3.1.3. Cytotoxicity of myristic acid analogs

Myristic acid analogs are generally considered to be nontoxic, to readily traverse the cell membrane, and to act as substrates for NMT. For some cellular *N*-myristoylproteins, incorporation of these analogs leads to analog-specific and analog-

dependent redistribution from membrane to cytosolic fractions. This dual level of selectivity, which encompasses selective incorporation and selective perturbation of function, probably accounts for their lack of cellular toxicity (Devadas *et al.*, 1992). Some myristic acid analogs, such as 12-methoxydodecanoic acid **66e** and 12-azidododecanoic acid **66h**, inhibit HIV-1 replication in acutely and chronically infected human T-lymphocyte cell lines at doses which do not cause cellular toxicity (Langner *et al.*, 1992). 12-Methoxydodecanoic acid (**66e**) thus selectively interferes with HIV myristoylated proteins yet produces minimal changes in the endogenous myristoylated proteins found in the host cells (Pidgeon *et al.*, 1993).

Cytotoxicity assays were performed using different cell lines and methods. The KB cell line was used for *in vitro* cytotoxicity assessment. Toxicity (TD<sub>50</sub> in mM) was determined for selected compounds (Table 3.8).

**Table 3.8.** *In vitro* cytotoxicity of some fatty acids in the KB cell line.

| Compound                | TD <sub>50</sub> (mM) <sup>a</sup> |
|-------------------------|------------------------------------|
| <b>66d</b>              | 0.17                               |
| <b>66e</b>              | 0.19                               |
| <b>67b</b>              | 0.19                               |
| <b>68c</b>              | 0.15                               |
| <b>69</b>               | 0.18                               |
| Adriamycin <sup>b</sup> | 0.00002                            |

<sup>a</sup> The concentration of the test compound that was cytotoxic to 50% of the cells. The value is the average of three separate experiments.

<sup>b</sup> Adriamycin as the reference drug.

*In vitro* cytotoxicity data acquired using a KB cell line (Parang *et al.*, 1996) are in agreement with the reported data since none of the myristic acid analogs tested (**69**, **66d**, **66e**, **67b**, **68c**) exhibited cytotoxicity (TD<sub>50</sub> = 0.15-0.19 mM). In the case of (±)-2-bromotetradecanoic acid (**68c**), the TD<sub>50</sub> concentration in the KB cell line is 4-12 fold higher (Table 3.8) than its antifungal MIC value in RPMI 1640 growth medium



(Table 3.3).

Cytotoxicity assay data from the CD4 lymphocyte CEM cell line ( $IC_{50} > 200 \mu M$  for 66a, 66j and 67f) and 2.2.15 cell line ( $IC_{50}$  for 66e =  $378 \mu M$ ) confirmed these conclusions. Although compound 66f is only a moderately active anti-HIV agent, its antiviral activity is not believed to be the result of cytotoxicity since more cytotoxic compounds such as 66g, 67d and 66h do not exhibit comparable antiviral activity (Parang *et al.*, 1996). 12-Halododecanoic acids such as the 12-bromo- and 12-iodo-analogs showed both a very low toxicity, and an increased percentage of surviving HIV-infected cells (71-79%) at 60-70  $\mu M$ .

Inhibition of HBV DNA replication by 12-methoxydodecanoic acid 66e in 2.2.15 cell culture (HBV RI intracellular DNA, HBV virion extracellular DNA) showed less toxicity compared to the reference compound ddC. However, the selectivity index and cytotoxicity for 66e are much lower than that for 3TC (Table 3.6).

### 3.3.2. Prodrug esters (81a-87, 91a-93)

#### 3.3.2.1. Biological activity of prodrug esters (81a-87, 91a-93)

##### 3.3.2.1.1. Anti-HIV activity of 5'-O-myristoyl analogue derivatives of AZT, FLT, dThd

In order to gain a better insight into the structural requirements for 2',3'-dideoxy nucleoside analogs with respect to optimal anti-HIV activity, the 5'-O-myristoyl analog esters of AZT (3'-N<sub>3</sub>), FLT (3'-F) and dThd (3'-OH) were synthesized. The 5'-O-myristoyl ester prodrug of AZT, FLT and dThd are presented in Table 3.2.

All of the 5'-O-myristoyl derivatives were evaluated by the U.S. National Cancer Institute Antiviral Evaluations Branch in an *in vitro* anti-HIV screen using HIV-1 infected CD4 lymphocytes (CEM cell line). Data for the ester prodrugs were compared with the corresponding test results for AZT which served as the treated control, performed at the same time.

### 3.3.2.1.1.1. Activity of 5'-O-myristoyl analogue derivatives of thymidine against human immunodeficiency virus (HIV)

The treatment of established cell lines such as H9 and U937 with deoxythymidine (dThd) (8) prior to infection results in a dramatic reduction in virus production. Thus, exposure to dThd (8) produced a 5- to 10-fold reduction in deoxycytidine triphosphate (dCTP), and a corresponding increase in thymidine triphosphate (TTP) levels (Meyerhans *et al.*, 1994). It therefore appears that dThd (8) influences HIV replication via modulation of intracellular deoxynucleotide pools (dNTP), particularly dCTP pools. The observation that inhibition of HIV-1 replication by dThd is reversed by compounds known to restore the dNTP pool such as deoxycytidine (dCyd), is evidence that the inhibitory effects exhibited by dThd against HIV occurs via modulation of dNTP pools (Meyerhans *et al.*, 1994). The addition of thymidine to cells significantly enhances the phosphorylation of ddC, presumably by reducing the intracellular pools of cytidine deoxynucleotides (Balzarini *et al.*, 1987a).

Acyl derivatives of uridine, cytidine, or deoxycytidine are effective in the treatment of hematopoietic toxicity induced by AZT. In some cell cultures, dThd was found to attenuate the toxicity (Borstel and Reid, 1993). However, administration of dThd to modify AZT chemotherapy in the clinical setting is neither practical (prolonged infusion of dThd), or satisfactory (orally administered thymidine is poorly absorbed) (Borstel and Reid, 1993). dThd has a short plasma half-life (8-10 min), due to its rapid hepatic catabolism to thymine and then eventually to  $\beta$ -aminoisobutyric acid and CO<sub>2</sub> (Ensminger and Rosowsky, 1979). As with other rapidly cleared drugs, a reasonable alternative to achieve sustained high blood levels would be to administer dThd as an ester derivative, assuming that chemical and/or enzymatic cleavage of the ester prodrug occurs at an appropriate rate. For example, administration of the 5'-O-pivaloate derivative of dThd to rats resulted in an increased uptake of the nucleoside, and a slower rate of elimination (Rosowsky *et al.*, 1981). Some acyl derivatives of dThd have now been designed to overcome the disadvantage of administering thymidine (8) in conjunction with other anti-HIV drugs. Administration of an acyl-thymidine derivative in combination with AZT may

be a useful method to prevent some toxic side effects such as hematopoiesis induced by AZT.

On the other hand, as discussed previously, myristoylation of eukaryotic viral proteins by myristoyl CoA: protein *N*-myristoyltransferase (NMT) has been observed for a number of DNA and RNA virus families (Chow and Moscufo, 1993). Addition of a tetradecanoyl (myristoyl) group is essential for assembly/replication of human immunodeficiency virus I (HIV-1). Some fatty acids such as 12-bromododecanoic acid and 4-oxatetradecanoic acid have shown promising activity against HIV-infected CD4 lymphocytes that was attributed to biochemical effects on HIV proteins (Pr55<sup>gag</sup> and Nef) (Schultz *et al.*, 1983b; Chow and Moscufo, 1993, Parang *et al.*, 1996) at concentrations which do not cause cellular toxicity (Langner *et al.*, 1992). These compounds may serve as alternative substrates for NMT. Despite their efficacy, their utility is limited by a high lipid solubility and rapid enzyme degradation. In general, fatty acids, such as myristic acid, are metabolized by  $\alpha$ -,  $\beta$ -, and  $\omega$ -oxidation (Klein *et al.*, 1971), and are ineffective for systematic use, in spite of their low toxicities, because they are metabolized by the host via the usual fatty acid pathways (Gershon *et al.*, 1978a-c). Accordingly, 5'-monoesters of thymidine (dThd) may offer a method to deliver myristic acid analogs more efficiently. Combinations of drugs that inhibit different targets, however, often offer surprising potency, even though one or more of the constituent compounds may be weakly active.

The biological properties of 5'-*O*-myristoyl ester derivatives of dThd were designed to improve delivery, and decrease the catabolism, of both dThd and the myristic acid analogs in an attempt to improve their anti-HIV activity, are now reported. *In vitro* anti-HIV-1 structure-activity relationships for these thymidine esters reveals unexpected anti-HIV-1 activities. Correlations between anti-HIV-1 activity of the fatty acids and their corresponding 5'-*O*-thymidine esters were investigated with respect to stability of the esters to porcine liver esterase and conformational features.

### 3.3.2.1.1.1. Anti-HIV activity of 5'-*O*-myristoyl analogs of thymidine

These 5'-*O*-acyl analogs of thymidine were designed to act as prodrugs to improve the delivery and delay the catabolism of both NMT-active fatty acids and thymidine for

enhancement of their individual anti-HIV activity. The anti-HIV-1 test data and the rates of *in vitro* ester hydrolysis for **81a-h** by porcine liver esterase at 37 °C are presented in Tables 3.9 and 3.17. Since many of these myristoyl analogs exhibited weak anti-HIV activity, the  $EC_{50}$ ,  $IC_{50}$  and selectivity index could not be determined. Accordingly, inhibition of viral replication (% protection) was scored as a percentage reduction in the virus-induced cytopathic effect (CPE, Table 3.9). Cytotoxicity ( $IC_{50}$ ) values for the test compounds, and the test compound concentration required to provide the specified level of protection to infected CD4 lymphocytes, are presented in Table 3.9. Among the 5'-*O*-acyl derivatives of thymidine, three compounds (**81d**, **81e** and **81h**) exhibited moderate activity. Structure-activity data revealed that the nature of the 5'-*O*-acyl substituent (**81a-h**) is a determinant of anti-HIV-1 activity. There was some but not significant correlations between that anti-HIV activity for the fatty acids **66d**, **67b**, **66h** and their corresponding 5'-*O*-esters (**81d**, **81e** and **81h**) when the *in vitro* anti-HIV assay was carried out using the same method and conditions. For example, 5'-*O*-(12-azidododecanoyl)thymidine (**81h**) was the most active ester ( $EC_{50} = 4.6 \pm 2.7 \mu M$ , 50% of maximum protection), in contrast to the weak activity exhibited by the corresponding 12-azidododecanoic acid (**66h**) which produced  $27 \pm 1.3\%$  of maximum protection against HIV-1 infected T lymphocytes at a 20  $\mu M$  concentration (Parang *et al.*, 1997a), and 5'-*O*-(11-thioethylundecanoyl)thymidine (**81e**) which provided 50% protection at 57.5  $\mu M$  ( $EC_{50} = 57.5 \pm 4.5 \mu M$ ) compared to the  $33.1 \pm 3.1$  maximum protection provided by 11-thioethylundecanoic acid (**67b**) at 63  $\mu M$  (Parang *et al.*, 1996). On the other hand, 5'-*O*-(12-bromododecanoyl)thymidine (**81d**) and 12-bromododecanoic acid (**66d**) showed  $EC_{50} = 25.7 \pm 7.6 \mu M$  and  $EC_{50} = 38.6 \pm 2.7 \mu M$  values, respectively (Parang *et al.*, 1996). The ester derivatives (**81a-h**) are generally more cytotoxic than the corresponding fatty acids. For example, the  $IC_{50}$  values for the 11-bromo ester (**81c**) and 12-bromododecanoic acid are 54  $\mu M$  and 165  $\mu M$ , respectively (Parang *et al.*, 1996).

The absence of significant correlation between the anti-HIV activity exhibited by the thymidine esters (**81d**, **81e** and **81h**) with the anti-HIV activity for the corresponding fatty



**Table 3.9. Anti-HIV activity of 5'-O-acyl derivatives of thymidine (81a-h).**

| No. | IC <sub>50</sub> (μM) <sup>a</sup> | Concentration (μM) <sup>b</sup>     |            |             |             |                 |
|-----|------------------------------------|-------------------------------------|------------|-------------|-------------|-----------------|
|     |                                    | 0.2                                 | 2.0        | 6.4         | 20.0        | 52.0            |
|     |                                    | Percent Protection (%) <sup>c</sup> |            |             |             |                 |
| 81a | 12.0 ± 0.0                         | 25.3 ± 2.6                          | 13.7 ± 3.8 | 7.9 ± 3.4   | 6.2 ± 4.1   | 7.8 ± 1.1       |
| 81b | 18.0 ± 0.0                         | 22.8 ± 5.3                          | 22.2 ± 1.4 | 12.5 ± 2.9  | 9.9 ± 3.6   | 5.4 ± 0.3       |
| 81c | 54.0 ± 1.4                         | 7.3 ± 0.2                           | 10.4 ± 3.3 | 9.0 ± 0.3   | 12.2 ± 1.5  | 8.6 ± 1.0       |
| 81d | > 52                               | 13.4 ± 2.8 <sup>d</sup>             | 12.4 ± 1.7 | 30.6 ± 6.1  | 45.1 ± 7.3  | 75.2 ± 7.2      |
| 81e | 41.0 ± 1.0                         | 17.3 ± 3.8 <sup>e</sup>             | 18.2 ± 4.2 | 17.4 ± 8.1  | 42.7 ± 10.2 | 47.0 ± 4.2      |
| 81f | > 20                               | 12.2 ± 3.2                          | 9.6 ± 2.3  | 11.0 ± 1.9  | 10.9 ± 0.7  | ND <sup>f</sup> |
| 81g | 32.0 ± 6.5                         | 6.0 ± 1.7                           | 6.5 ± 1.6  | 12.8 ± 6.3  | 16.3 ± 4.6  | 28.8 ± 0.4      |
| 81h | > 21                               | 17.4 ± 7.4 <sup>g</sup>             | 24.9 ± 5.6 | 55.6 ± 18.9 | 56.3 ± 8.2  | ND              |
| AZT | 100                                | EC <sub>50</sub> = 0.01             |            |             |             |                 |

<sup>a</sup> IC<sub>50</sub> is defined as the test compound concentration required to reduce the number of viable untreated CD4 lymphocytes by 50%. <sup>b</sup> Concentration of the test compound that produces the percentage protection specified against HIV infected T-lymphocytes. <sup>c</sup> The percentage of surviving HIV-infected cells treated with the test compound (at the concentration indicated) relative to uninfected, untreated controls (the value is the average of two separate experiments). <sup>d</sup> (EC<sub>50</sub> = 25.7 ± 7.6) (TI<sub>50</sub> > 2.0), EC<sub>50</sub> is defined as the 50% antiviral effective concentration, required to produce a 50% reduction of the cytopathic HIV effect in CD4 lymphocytes (mean ± SEM, n = 4). TI<sub>50</sub> is defined as the therapeutic index (IC<sub>50</sub> / EC<sub>50</sub>) (mean ± standard error, n = 4). <sup>e</sup> (EC<sub>50</sub> = 57.5 ± 4.5) (TI<sub>50</sub> = 0.73 ± 0.08). <sup>f</sup> ND = Not determined. <sup>g</sup> (EC<sub>50</sub> = 4.6 ± 2.7) (TI<sub>50</sub> > 4.66).

acids suggests that different mechanisms may be responsible for the anti-HIV activity of 5'-O-ester derivatives of thymidine (81a-h). For example, 81a-h may exert their anti-HIV effect as the intact ester, rather than as the hydrolyzed myristic acid species. Therefore, the stability of the 12-azidododecanoyl ester (81h) was determined in the same culture medium (RPMI 1640/10% FBS) used in the NIH anti-HIV-1 assay (Weislow *et al.*, 1989). The hydrolysis reaction, which followed first order kinetics, showed that 81h is



relatively stable, although it had undergone partial hydrolysis in culture medium after 6 days ( $t_{1/2} = 8.02$  days). Most of the compound **81h** (60%) remained intact in the culture medium after incubation for six days. Similar stabilities are expected for the other structurally related ester compounds investigated. Additional studies are required to clarify the mechanism of action of these ester analogs (**81d**, **81e** and **81h**) in the CEM cell line, to determine their hydrolysis in the cell culture medium, and to explain their differences in activity relative to the corresponding fatty acids (**66d**, **67b** and **66h**). It is possible that the esters (**81d**, **81e** and **81h**) exert their anti-HIV activity after incorporation into cells prior to hydrolysis to the corresponding fatty acids. Another possible explanation for the differences in anti-HIV activity between the myristic acid, and myristoyl ester, analogs could be differences in their rate of uptake into cells. It has been shown that replacement of one or more methylene groups in myristic acid by a halogen, sulfur or an azide exerts a substantial effect on antiviral activity, and that selectivity with respect to the nature and position of the heteroatom is observed. A general conclusion emerging from this work is that small variations in the structure of the 5'-O-acyl prodrug moiety can produce significant changes in the anti-HIV activity exhibited by 5'-O-acyl derivatives of thymidine.

These results have confirmed the importance of an azido group at the 3'-position of AZT for efficacy in the nanomolar range, since none of 5'-O-myristoyl thymidine analogs showed potent activity in this range. The ester **81d** was the most active analog with an  $EC_{50} = 4.6 \mu\text{M}$ . Also, the importance of the 12-position in the thio analog (**81e**), the 12-azido analog (**81h**), and the 12-bromo analog (**81d**) is clear since all exhibited moderate activity.

#### 3.3.2.1.1.2. Cytotoxicity of 5'-O-myristoyl analogue derivatives of dThd

Since many anti-HIV agents do not produce complete viral suppression, the design of inhibitors with different toxicity profiles, either alone or in combination, has been a high clinical research priority. The antiviral activity exhibited by compounds **81d**, **81e** and **81h** is not likely to be the result of cytotoxicity since more cytotoxic compounds such as **81a** and **81b** do not exhibit comparable antiviral activity. These *in vitro* results are indicative of

a very low toxicity for some analogs ( $IC_{50} > 52 \mu M$ ) (Table 3.9).

### 3.3.2.1.1.1.3. Physicochemical properties of 5'-O-myristoyl analogue derivatives of thymidine

#### 3.3.2.1.1.1.3.1. Conformational analysis

The molecular conformation of nucleosides has been reviewed extensively, primarily by Saenger *et al.*, (1984). Parameters that describe the most important features of nucleoside conformation include the geometry of the glycosyl link (torsion angle  $\chi$ , C2-N1-C1'-O4'), the furanose ring puckering (pseudo-rotation angle  $\rho$ ), the torsion angle C6'-O5'-C5'-C4' ( $\phi$ ) and the orientation of the 5'-hydroxyl group (torsion angle  $\gamma$ , C3'-C4'-C5'-O5'). Detailed analyses of the conformational properties of a number of antiviral nucleosides were directed at identifying conformational features that may correlate with activity (Nasr *et al.*, 1990; Chu *et al.*, 1989; Van Roey *et al.*, 1989; Camerman *et al.*, 1984). The goal of this study was to analyze the conformational properties of nucleoside esters (**81a-h**) and to determine if their observed conformations and associated energy profiles could contribute to an understanding of their biological activities.

Some important structural features of the optimized structures for compounds **81a-h** such as dihedral and torsion bond angles are presented in Table 3.10. MM<sup>+</sup> molecular mechanics geometry optimization (Allinger, 1977), followed by atomic charge assignment derived from PM3 single point calculations or PM3 full geometry optimization (Stewart, 1989), were used to obtain the lowest energy conformer, among a number of possible conformers, by inspection of their energy distributions. In these conformers, the furanose ring was puckered in a C(2')-endo/C(3')-exo conformation. All esters (**81a-h**) have similar conformations for the nucleoside moiety, with calculated MM<sup>+</sup> molecular mechanics energies of  $-51.20 \pm 4.48$  kcal/mole and PM3 semi-empirical energies of  $-6738.58 \pm 361.61$  kcal/mole. Moderately active analogs (**81d**, **81e** and **81h**) did not show significant energy differences, relative to less potent compounds (**81a-c**, **81f** and **81g**), which may be correlated with their greater anti-HIV-1 activity. The optimized conformations for **81a** is shown in Figure 3.3.

These studies revealed that the moderately active compounds **81d**, **81e**, and **81h** adopt

a furanose ring conformation similar to other ester analogs (C-2' endo/C3'-exo, where endo and exo refer to displacements of the atoms above and below the plane of the ring, respectively).

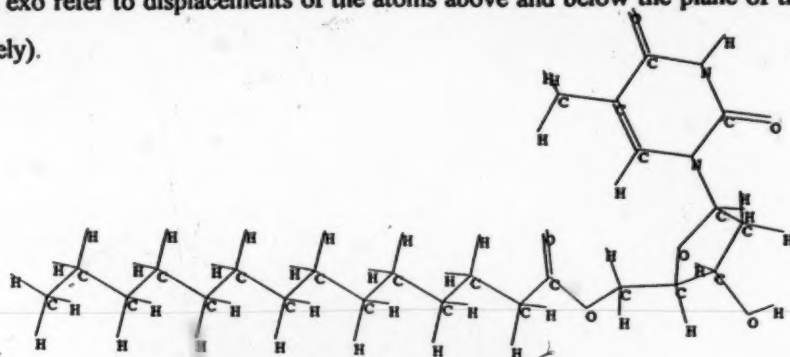


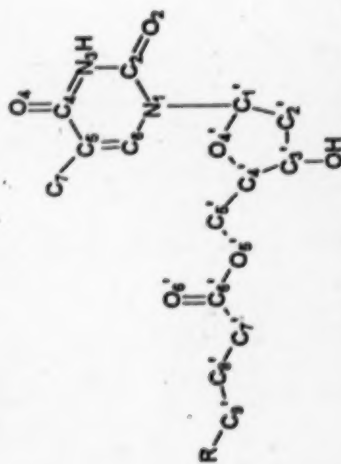
Figure 3.3. Minimized structure for 81a.

All of the esters investigated were found to have a similar C-2' endo conformation for the sugar moiety, and a similar energy for the lowest energy conformer. Derived data for bond lengths, dihedral and torsion bond angles indicate that the 5'-O-acyl substituent has little influence on the conformation of the nucleoside (thymidine) moiety. The dihedral and torsion angles are similar for the nucleoside moiety of 81a-h, but they differ for the heteroatom centers in the fatty acid moiety. It was not possible to draw any correlations between properties derived from the molecular mechanics calculations and anti-HIV-1 activity due to the small changes introduced after esterification [std. dev. = 0.01-2.85 ( $^{\circ}$ )].

#### 3.3.2.1.1.3.2. Correlation of partition coefficient with anti-HIV activity of 5'-O-myristoyl analogue derivatives of thymidine

Apparent and estimated partition coefficients (Log P) for the 5'-O-myristoyl analogue derivatives of thymidine (81a-h) were determined (see Table 3.15.1). There appears to be a direct correlation between anti-HIV activity and Log P for these compounds (81a-h), since the anti-HIV data suggest that the experimental partition coefficient should be in the Log P 4.6-4.8 range for optimal anti-HIV activity (see compounds 81d, 81e and 81h in Table 3.15.1). The contribution of partition coefficient in the QSAR regression equation

Table 3.10. Selected dihedral and torsion bond angles (°) for 5'-O-acyl derivatives of thymidine (81a-h) after optimization by MM<sup>+</sup> molecular mechanics, and PM3 semi-empirical, calculations.



| Dihedral angle (°) <sup>a</sup> | 5'-O-Acetylthymidine | Esters 8a-h <sup>b</sup> | Dihedral angle (°) <sup>a</sup> | 5'-O-Acetylthymidine | Esters 8a-h <sup>b</sup> |
|---------------------------------|----------------------|--------------------------|---------------------------------|----------------------|--------------------------|
| O6-N1-C1'                       | 121.58               | 121.67 ± 0.30            | C2-N1-C1'-O4'                   | -135.05              | -145.85 ± 0.89           |
| C1'-O4'-C4'                     | 108.96               | 109.06 ± 0.17            | O3'-C3'-O4'-C3'                 | -168.72              | -176.67 ± 0.38           |
| N1-C1'-C2'                      | 113.22               | 113.05 ± 0.47            | O6'-O5'-C3'-O4'                 | -108.95              | -103.67 ± 1.43           |
| C1'-C2'-C3'                     | 100.44               | 100.46 ± 0.06            | C1'-C2'-C3'-O3'                 | 84.16                | 83.77 ± 0.85             |
| C2'-C3'-O3'                     | 108.84               | 108.72 ± 0.36            | O3'-C3'-O4'-O4'                 | -109.83              | -100.23 ± 0.46           |
| O4'-O4'-C3'                     | 107.52               | 107.50 ± 0.10            | O4'-C1'-N1-C6                   | 38.09                | 36.73 ± 0.74             |
| C3'-O4'-C3'                     | 112.81               | 112.82 ± 0.05            | C3'-C1'-N1-C6                   | -82.33               | -82.16 ± 0.79            |
| C3'-O3'-C6'                     | 119.37               | 119.42 ± 0.13            | C3'-C4'-C3'-O3'                 | -168.72              | -176.67 ± 0.38           |
| O5'-O6'-C7'                     | 108.96               | 109.12 ± 0.22            | O4'-O4'-C1'-C2'                 | -13.99               | -30.78 ± 0.43            |
| C2-N1-C1'                       | 120.35               | 120.25 ± 0.30            | C1'-C2'-C3'-O4'                 | 20.24                | 38.82 ± 0.24             |
| N1-C1'-O4' (θ)                  | 112.10               | 112.07 ± 0.08            | C1'-C2'-C3'-O4'                 | -18.20               | -31.56 ± 0.20            |
| O4'-C1'-C2'                     | 105.23               | 105.20 ± 0.20            | C2'-C3'-O4'-C1'                 | 10.86                | 15.22 ± 0.45             |
| C2'-C3'-O4'                     | 100.44               | 102.14 ± 1.11            | C3'-O4'-O4'-C1'                 | 1.81                 | 9.63 ± 0.53              |
| O4'-C1'-C2'                     | 109.05               | 109.03 ± 0.03            | O6-N1-C1'-C2'                   | -82.14               | -82.16 ± 0.79            |
| O4'-C1'-C2'                     | 107.99               | 108.03 ± 0.39            | O4'-O4'-C1'-N1'                 | 36.71                | -36.73 ± 0.75            |
| O4'-C1'-C2'                     | 109.11               | 108.92 ± 0.35            | C2'-C1'-O4'-C4'                 | -154.50              | -154.01 ± 1.00           |
| O4'-C1'-C2'                     | 125.60               | 125.54 ± 0.15            | C3'-C3'-O4'-C3'                 | -13.99               | 30.78 ± 0.43             |
| O6'-O6'-C7'                     | 125.44               | 125.34 ± 0.24            | N1-C1'-C2'-C3'                  | -110.53              | -103.70 ± 0.52           |
| N1-C2-N3                        | 116.91               | 116.86 ± 0.27            | N1-C1'-O4'-C4'                  | 140.63               | 161.43 ± 0.27            |
| C3-N1-C6                        | 118.03               | 118.89 ± 1.75            | O3'-C3'-O4'-C3'                 | -137.40              | -154.32 ± 0.41           |
| O4-C5-C7                        | 121.70               | 121.65 ± 0.14            | C3'-C3'-O4'-C3'                 | 128.78               | 140.88 ± 0.53            |
| C4-C5-C7                        | 121.66               | 121.64 ± 0.05            | C2-N1-C1'-C2'                   | 95.31                | 95.26 ± 0.93             |
| N1-C2-O2                        | 123.55               | 123.50 ± 0.14            | O4'-O4'-C3'-O3'                 | 71.30                | 64.72 ± 0.56             |
| O3-C2-N3                        | 119.54               | 119.55 ± 0.04            | C5'-O5'-O6'-C7'                 | 175.10               | 179.34 ± 0.62            |
| C2-N3-O4                        | 122.39               | 119.55 ± 0.04            | O5'-O6'-C7'-C8'                 | -168.72              | 175.34 ± 2.39            |
|                                 |                      | 122.39 ± 0.01            | C3'-O4'-C3'-O5' (γ)             | -168.72              | -176.67 ± 0.38           |
|                                 |                      |                          | O5'-C7'-C8'-O9'                 | -177.32 ± 2.85       | 177.32 ± 2.85            |

<sup>a</sup>Some specific dihedral and torsion bond angles (°) for 81a-h: C-C-C-Br (81c) = 179.95 and (81d) = 179.98; C-S-C (81e) = 98.01; C-N-N-N (81f) = -179.99, (81g) = -179.97 and (81h) = -179.85; dihedral angle O-C-C-Br (81b) = 109.12. <sup>b</sup>Mean ± std. dev., n = 8, calculated for the 8 esters (81a-h).



confirms the importance of this descriptor with respect to anti-HIV-1 activity for these esters (81a-h).

### 3.3.2.1.1.3.3. Quantitative-structure-activity relationships (QSARs)

The minimum energy conformations were subsequently used in the *SciQSAR* program to obtain a regression equation.  $-\text{Log}(1/\text{PPR20})$  (Log Percent of protection against HIV-infected CD4 lymphocytes in the presence of 20  $\mu\text{M}$  test compound) was used as the QSAR parameter name and the experimental *n*-octanol/water partition coefficient (Log P) as a user-defined descriptor. The multiple correlation coefficient ( $R^2$ ) and the Fisher Statistic (F) were used to assess the quality of fit for the regression equation. Accordingly, a regression equation was established based on the anti-HIV-1 activity of compounds 81a-h where:

$$-\text{Log } 1/\text{PPR20} = 8.77 - 0.94 \text{ Log } P + 1.04 \times 10^{-3} \text{ WienI} + 0.0217 \text{ MW}$$

$$\text{RMSD} = 0.13, R^2 = 0.93, F = 26.57, n = 10$$

PPR20 = Log Percent of protection against HIV-infected CD4 lymphocytes in the presence of 20  $\mu\text{M}$  test compound, Log P = experimental *n*-octanol/water partition coefficient, WienI = Wiener Index, MW = Molecular Weight, RMSD = Root Mean Square,  $R^2$  = Multiple correlation coefficient, F = Fisher Statistic.

### 3.3.2.1.1.2. 5'-O-myristoyl analogue derivatives of AZT and FLT

#### 3.3.2.1.1.2.1. Anti-HIV activity of 5'-O-myristoyl analogue derivatives of AZT

Several novel ester prodrugs of zidovudine (82a-87) were synthesized in combination with a variety of fatty acids. The structures of the 5'-substituted AZT derivatives and the *in vitro* antiviral and cytotoxic activities of the conjugates of AZT are depicted in Table 3.11. The indicated values represent data, obtained for at least three separate determinations. The anti-HIV activity for AZT in HIV-infected CD4 lymphocytes using CEM cells reported previously by NIH was  $\text{EC}_{50} = 10 \text{ nM}$  and  $\text{IC}_{50} = 100 \mu\text{M}$ , and *in vitro* therapeutic index ( $\text{TI}_{50} = \text{IC}_{50}/\text{EC}_{50}$ ) was 10000. AZT and FLT were included as reference compounds in Table 3.11. It is interesting that these ester prodrugs retained the



replication inhibiting activity of the parent nucleoside when tested in *in vitro* assays for virus replication, although all of the derivatives (82a-87) were 5'-hydroxy esters. For AZT, it is known that the intracellular biologically active species is triphosphorylated at the 5'-hydroxy position. AZT, lacking a 5'-triphosphate group, is inactive against viral polymerases.

In general, the anti-HIV activities and cytotoxicities for these analogs of AZT were related to the activity of the parent compounds, their uptake rate into cells and their conversion rate to AZT. There are four possible explanations as to the mode of action of these 5'-substituted compounds. (a) The most likely is that the fatty acid group is rapidly cleaved from the nucleoside by any of a variety of hydrolytic enzymes present in the cell cytoplasm. The deacylated parent nucleoside could then be a substrate for phosphorylation that is required to generate the active triphosphate species. Therefore, the rate of intracellular hydrolysis would determine the amount of AZT released and ultimately the anti-HIV activity exerted by the prodrug. (b) The partially released fatty acids from these prodrugs may also produce a synergistic or an additive effect. (c) The rate of uptake of prodrugs into cells is an important factor in demonstrating their anti-HIV activity. A slower rate of uptake can cause a lower concentrations of prodrug and AZT hydrolytically-released inside the cells. (d) The possibility that the myristoyl moiety in some way substitutes for the usual phosphorylation in the myristoyl-nucleoside, resulting in a compound which is active without cleavage and phosphorylation (Horrobin *et al.*, 1994) is less likely. Based on results obtained some general trends pertaining to structure-activity relationships are evident:

(1) Structure function analysis revealed that the anti-HIV activity of the 5'-substituted derivatives is clearly dependent on the nature of the 5'-substituent. 3'-Azido-2',3'-dideoxy-5'-O-(4-oxatetradecanoyl)thymidine (82c) ( $EC_{50} = 1.4$  nM), 3'-azido-2',3'-dideoxy-5'-O-(9-thiatetradecanoyl)thymidine (82e) ( $EC_{50} = 5.5$  nM), 3'-azido-2',3'-dideoxy-5'-O-(12-bromododecanoyl)thymidine (83d) ( $EC_{50} = 3.2$  nM) and 3'-azido-2',3'-deoxy-5'-O-(tetradecanoyl)thymidine (87) ( $EC_{50} = 3.8$  nM) demonstrated a higher potency than AZT and other related analogs.

The ester prodrug 82c was approximately 7 times more potent than AZT in inhibiting

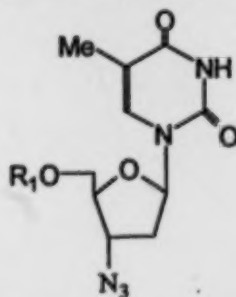
the viral replication (Figure 3.4). These results suggest that the increased inhibition of HIV-1 replication by compound 82e may be due to a higher intracellular level of AZT that is achieved for the prodrug relative to that for AZT. The high activity for 82e was possibly due to its increased rate of uptake and intracellular hydrolysis to the two antiviral agents AZT (6) and 4-oxatetradecanoic acid (65c). The inability of 84d, 84e and 85b to enhance antiviral activity may be due to incomplete intracellular hydrolysis of the prodrugs under the *in vitro* assay conditions.

To obtain a better insight into the presumed hydrolytic activation of these derivatives, incubation in CEM culture is required. The 5'-O-myristoyl analog (87) is more potent than AZT. The precise metabolic reason for the increased activity may be due to increased cellular uptake of the ester prodrug relative to that of AZT. The 2-fluoro analog (85a) is highly labile and probably liberates the nucleoside, at least in part, prior to membrane penetration.

(2) Several 5'-substituted AZT derivatives such as 84a, 84c and 85a demonstrated activities that were equivalent to that of reference drug (AZT, 6) against HIV-1, suggesting that these esters are readily hydrolyzed to release the parent compound AZT. These results indicate that the nature of a substituent(s) on the fatty acid chain is a determinant of antiviral activity, since other esters showed higher (82c, 82e, 83d, 84c, 87, 91b, 91d, 92) or lower (82a, 82b, 82d, 83a-c, 83e-i, 84b, 84d-f, 85b, 86, 91a, 91c, 93) potency against HIV-1 compared to AZT (Tables 3.11 and 3.12, Figure 3.4). The decreased activities observed for certain analogs may be due to slower regeneration of the parent nucleoside. Therefore, cleavage of the conjugate might have resulted in an *in vitro* potency by the conjugate that is weaker than that of the nucleoside.

(3) Introduction of a phenyl or phenoxy group (83g, 83i, 84d and 84e) on C-11 or C-12 of the chain induced a marked decrease in the inhibitory activity of these derivatives. The inability of 84d to increase antiviral activity may be due to incomplete intracellular hydrolysis of the prodrug under the *in vitro* assay conditions. The derivatives 85b and 86 also showed weak anti-HIV activity. The reason for the lower potency of the 2-bromo ester (85b) may reflect slower uptake into cells and/or a slow hydrolysis rate since the initial cellular uptake of the ester and cleavage of the ester is required for release of the

Table 3.11. Comparative potency and selectivity of 5'-O-myristoyl analogue derivatives of AZT as inhibitors of HIV replication in CD4 cells (CEM-SS cell line).



| Compound | R <sub>1</sub>                                                                      | IC <sub>50</sub> , μM <sup>a</sup> | EC <sub>50</sub> , nM <sup>b</sup> | TI <sub>50</sub> <sup>c</sup> |
|----------|-------------------------------------------------------------------------------------|------------------------------------|------------------------------------|-------------------------------|
| 82a      | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO               | 65.6±2.2                           | 82.6±35.7                          | 794.2                         |
| 82b      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO  | 64.1±0.1                           | 102.9±45.7                         | 622.4                         |
| 82c      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO | 31.5±1.25                          | 1.4±0.1                            | 22500.0                       |
| 82d      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> O(CH <sub>2</sub> ) <sub>7</sub> CO | 67.5±3.75                          | 17.0±5.0                           | 3970.6                        |
| 82e      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> S(CH <sub>2</sub> ) <sub>7</sub> CO | 100.5±4.75                         | 5.5±0.0                            | 18440.4                       |
| 83a      | F(CH <sub>2</sub> ) <sub>11</sub> CO                                                | >101                               | 124.0±21.9                         | 814.5                         |
| 83b      | I(CH <sub>2</sub> ) <sub>11</sub> CO                                                | 100.0±0.0                          | 49.5±5.3                           | 2020.2                        |
| 83c      | Cl(CH <sub>2</sub> ) <sub>11</sub> CO                                               | >110                               | 185.9±27.7                         | 591.7                         |
| 83d      | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | 37.0±0.5                           | 3.2±1.0                            | 11746.0                       |
| 83e      | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | >2                                 | 30.6±7.6                           | >65.5                         |
| 83f      | EtS(CH <sub>2</sub> ) <sub>11</sub> CO                                              | >2                                 | 130.8±26.3                         | >15.3                         |
| 83g      | Ph(CH <sub>2</sub> ) <sub>11</sub> CO                                               | 87.5±6.3                           | 105.5±0.02                         | 829.4                         |
| 83h      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | 160.0±0.0                          | 36.6±11.6                          | 4371.6                        |
| 83i      | PhO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | 88.2±5.8                           | 50.3±1.7                           | 1753.1                        |
| 84a      | MeO(CH <sub>2</sub> ) <sub>10</sub> CO                                              | 115.0±2.5                          | 8.8±0.0                            | 13068.2                       |
| 84b      | EtS(CH <sub>2</sub> ) <sub>10</sub> CO                                              | >2                                 | 48.9±10.6                          | >40.9                         |
| 84c      | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                               | 110±0.0                            | 7.3±1.9                            | 15068.5                       |
| 84d      | 4-I-PhO-(CH <sub>2</sub> ) <sub>10</sub> CO                                         | 180.5±13.3                         | 131.0±6.4                          | 1377.9                        |
| 84e      | PhO(CH <sub>2</sub> ) <sub>10</sub> CO                                              | 100.0±0                            | 47.5±2.8                           | 2105.3                        |
| 84f      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                                  | >6.5                               | 28.5±7.93                          | >228.4                        |
| 85a      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CHFCO                              | >56.1                              | 12.5                               | >4480                         |
| 85b      | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CHBrCO                             | 40.0±3.0                           | 127.5±41.3                         | 313.7                         |
| 86       | Ph(CH <sub>2</sub> ) <sub>9</sub> CO                                                | 110.0±1.0                          | 160.1±47.0                         | 687.3                         |
| 87       | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                                 | >2                                 | 3.8±0.3                            | >526                          |
| FLT      | H                                                                                   | 2.4 <sup>d</sup>                   | 2.5 <sup>d</sup>                   | 967                           |
| AZT      | H                                                                                   | 100.0                              | 10.0                               | 10000.0                       |
| 3TC      | 2',3'-dideoxy-3'-thiacytidine                                                       | >100 <sup>e</sup>                  | 0.01 <sup>f</sup>                  |                               |

<sup>a</sup>IC<sub>50</sub> is defined as the inhibitory concentration, required to reduce the number of viable untreated CD4

lymphocytes by 50% (mean ± SEM, n = 5). <sup>b</sup>EC<sub>50</sub> is defined as the 50% antiviral effective concentration, required to produce a 50% reduction in the cytopathic HIV effect in CD4 lymphocytes (mean ± SEM, n = 5).

<sup>c</sup>TI<sub>50</sub> is defined as the therapeutic index (IC<sub>50</sub>/EC<sub>50</sub>) (mean ± SE, n = 5). <sup>d</sup>FLT activity taken from Hiebl *et al.*, 1996 (CEM cell line). <sup>e</sup>3TC cytotoxicity adopted from Schinazi *et al.*, 1992a, in the CEM cell line. <sup>f</sup>3TC activity adopted from Balzarini *et al.*, 1996, in CEM/0 infected with HIV-1(III<sub>B</sub>).

AZT. This ester 85b, which is not very labile *in vitro*, would produce AZT very slowly since the highly sterically hindered environment in the region of the carboxy group makes enzymatic attack at the ester group difficult. Determination of the rates of enzymatic hydrolysis also showed a higher stability for these analogs compared to that of others (Tables 3.17.1 and 3.17.2, see section 3.3.2.3.2.2).

(4) The effect of a variety of substituents at C-12 of the acyl moiety was also evaluated. There were no dramatic differences in the anti-HIV activity between 82a and 82b. 3'-Azido-2',3'-dideoxy-5'-O-(12-azidododecanoyl)thymidine (83h) was less toxic than AZT. The placement of a polar group (azido, bromo, iodo, fluoro, and chloro) at position 12 of selected esters (83a, 83c, 83h) reduced the toxicity of the prodrug significantly. However, this was not the case for 82a and 82b that had oxygen in the acyl chain.

(5) The *n*-octanol/phosphate buffer partition coefficients of some derivatives were determined in order to compare their lipophilicities with AZT (Tables 3.15.1 and 3.15.2). For 5'-O-fatty acyl derivatives of AZT, optimum selectivity and potency was observed for analogs (82c, 82e, 83d) with partition coefficients in the range 5.67-6.24. For example, highly lipophilic compounds (83f, 83g, 83i, 84d, 84e, 85b, 86) (Log P > 6.6) are less potent and less selective than other analogs. Some analogs such as 87, which had partition coefficients >6.6, but exhibited greater activity suggesting that other factors such as ester hydrolysis rate and their uptake rate into the cells may also be involved in overall activity observed. Uptake of these compounds into cells could be related to their partition coefficient. Highly lipophilic compounds (Log P > 6.6) were less potent and less selective than other analogs. These observations indicated that some esters are either not taken up by the cells or not hydrolyzed to the corresponding nucleoside.

#### 3.3.2.1.1.2.1.1. Cytotoxicity of 5'-O-myristoyl analogue derivatives of AZT

The IC<sub>50</sub> (measure of its cellular toxicity to uninfected cells) for AZT in this system was 100  $\mu$ M, whereas for prodrugs it ranged from > 2 to 181  $\mu$ M. The IC<sub>50</sub> for 82c (IC<sub>50</sub> = 32  $\mu$ M) is less than that for AZT (IC<sub>50</sub> = 100  $\mu$ M). Compound 82c exhibited a higher



therapeutic index than AZT (22,500 vs 10,000). When toxicity is taken into account, the most selective compounds (higher therapeutic index) were 82c, 82e, 84a, and 84c.

The phenoxy derivative (84d) was less cytotoxic than AZT, suggesting that the slow release of AZT from the prodrugs may be related to the reduction in toxicity. Compound 84d was less susceptible to plasma esterases (Table 3.16.2) and was also less toxic than AZT under the *in vitro* assay conditions. However, the 2-3-fold increase in toxicity observed for 82c and 83d may be due to their higher uptake and more rapid hydrolysis in uninfected CD4 lymphocytes. Compounds 82c and 83d, which were 3-7-fold more active in inhibiting virus replication, were also approximately 3-fold more cytotoxic than AZT.

The reduced toxicity in the *in vitro* anti-HIV assay may be primarily due to slow intracellular hydrolysis of the prodrug to AZT. *In vitro* cell culture data showed that 85b was completely stable after six days of incubation in the RPMI/10% FBS culture medium used for the anti-HIV assay. Therefore, it is postulated that most of the prodrugs may remain as intact esters extracellularly.

#### 3.3.2.1.1.2.2. Anti-HIV activity of 5'-O-myristoyl analogue derivatives of FLT

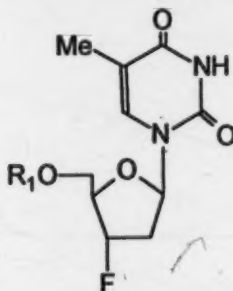
The ability of FLT prodrugs (91a-93) to protect CEM cells against HIV-induced cytopathogenicity was evaluated. FLT has been reported to be one of the most active inhibitors of HIV *in vitro* in various T-cells ( $EC_{50} = 1-10$  nM). On the basis of cell culture data, FLT is a more potent inhibitor of HIV replication than 3'-azido-3'-deoxythymidine (Balzarini *et al.*, 1988). Although FLT was more effective than AZT in inhibiting HIV cytopathogenicity in CEM cells, it proved to be considerably more cytotoxic than AZT toward uninfected lymphocytes. The selectivity index (SI) of AZT is much greater than that for FLT (10,000 vs 967). The clinical development of FLT has been discontinued. The observed side-effects for FLT stimulated the search for closely related nucleoside analogs with increased antiviral activity and decreased cytotoxicity (Hiebl *et al.*, 1996).

The results suggest that the low lipophilicity of FLT ( $P = 0.5$ ) may retard its entry into the brain by passive diffusion, resulting in an inadequate therapeutic brain concentration. An additional ester group should enhance the lipophilicity of FLT. This may facilitate entry into the brain by passive diffusion, and thus increase the otherwise inadequate therapeutic



drug-brain concentrations attained by FLT (Kumar *et al.*, 1994a). Therefore, it was of interest to prepare more lipophilic derivatives of FLT for anti-HIV evaluation.

**Table 3.12.** Comparative potency and selectivity of 5'-O-myristoyl analogue derivatives of FLT as inhibitors of HIV replication in CD4 cells (CEM-SS cell line).



| Compound | R <sub>1</sub>                                      | IC <sub>50</sub> , μM <sup>a</sup> | EC <sub>50</sub> , nM <sup>b</sup> | TI <sub>50</sub> <sup>c</sup> |
|----------|-----------------------------------------------------|------------------------------------|------------------------------------|-------------------------------|
| 91a      | Br(CH <sub>2</sub> ) <sub>11</sub> CO               | 29.0±3.0                           | 25.0±3.0                           | 1160.0                        |
| 91b      | MeO(CH <sub>2</sub> ) <sub>11</sub> CO              | 79.0±1.0                           | 3.8±1.5                            | 21050.6                       |
| 91c      | EtS(CH <sub>2</sub> ) <sub>11</sub> CO              | 33.0±0.0                           | 16.0±0.5                           | 2062.5                        |
| 91d      | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO  | 94.0±6.0                           | 2.8±1.0                            | 34181.8                       |
| 92       | EtS(CH <sub>2</sub> ) <sub>10</sub> CO              | 42.5±1.3                           | 6.0±1.8                            | 7036.4                        |
| 93       | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO | 120.0±0.0                          | 44.50±4.8                          | 2696.6                        |
| AZT      | H                                                   | 100.0                              | 10.0                               | 10000.0                       |
| FLT      | H                                                   | 2.4 <sup>d</sup>                   | 2.5 <sup>d</sup>                   | 967                           |

<sup>a</sup> IC<sub>50</sub> is defined as the inhibitory concentration, required to reduce the number of viable untreated CD4 lymphocytes by 50%. (mean ± SEM, n = 5). <sup>b</sup> EC<sub>50</sub> is defined as the 50% antiviral effective concentration, required to produce a 50% reduction in the cytopathic HIV effect in CD4 lymphocytes (mean ± SEM, n = 5). <sup>c</sup> TI<sub>50</sub> is defined as the therapeutic index (IC<sub>50</sub> / EC<sub>50</sub>) (mean ± SEM, n = 5). <sup>d</sup> FLT activity taken from Hiebl *et al.*, 1996 (CEM cell line).

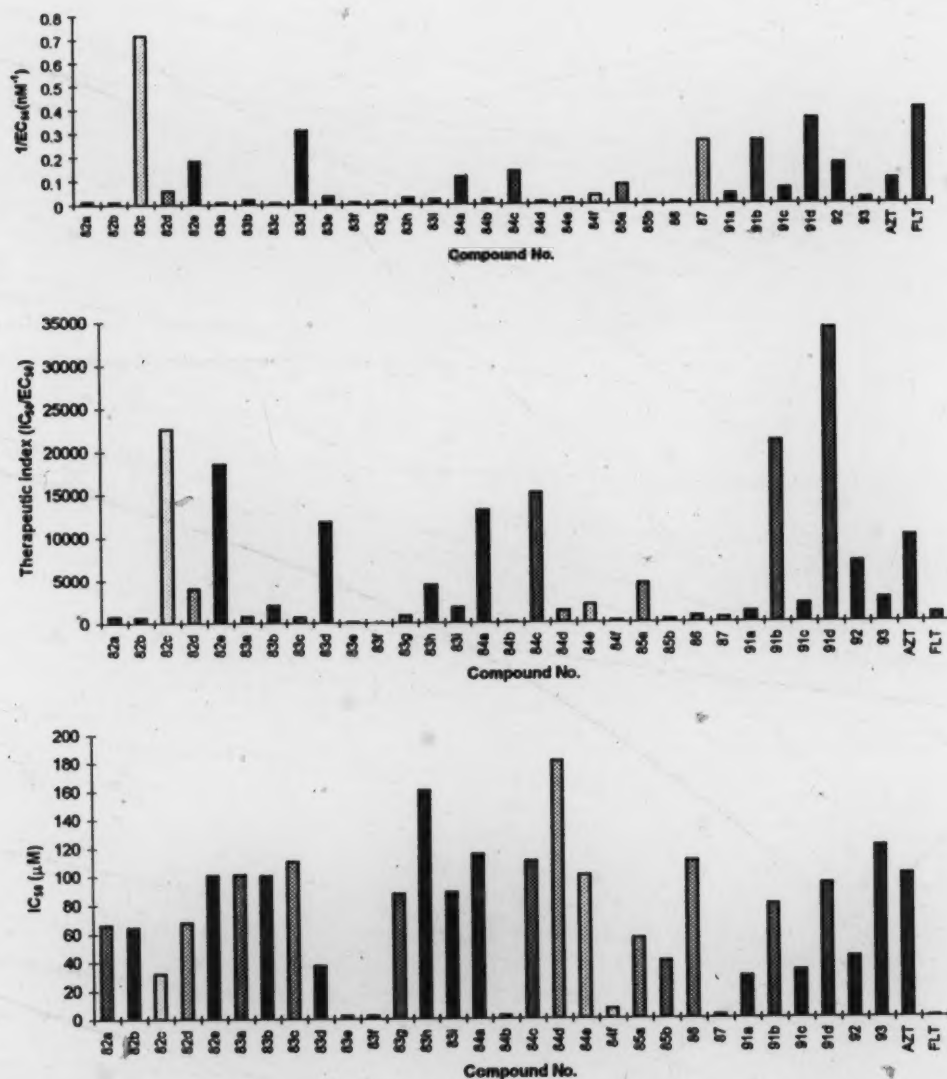


Figure 3.4. Comparative potency, toxicity and selectivity of 5'-O-myristoyl analogue derivatives of AZT and FLT.

Some general conclusions pertaining to structure-activity relationships for this category of compounds can be made on the basis of these results. The general trends described in section 3.3.2.1.1.2.1 for the structure-activity relationships of AZT derivatives can also be applied to explain the anti-HIV activity data for 5'-O-myristoyl analogue derivatives of FLT presented in Table 3.12 and Figure 3.4.

(1) It was found that two prodrugs of FLT, 3'-fluoro-2',3'-deoxy-5'-*O*-(12-methoxydodecanoyl)thymidine (**91b**) ( $EC_{50} = 3.8$  nM) and 3'-fluoro-2',3'-deoxy-5'-*O*-(12-azidododecanoyl)thymidine (**91d**) ( $EC_{50} = 2.8$  nM), were the most effective anti-HIV-1 agents in this group. The ester **91d** exhibited potency comparable to FLT (Table 3.12).

(2) 3'-Fluoro compounds **91b-92** were more potent than their corresponding 3'-azido derivatives (**83e**, **83f**, **83h**, **84b**) (See Figure 3.4) probably due to a higher hydrolysis rate of the 3'-fluoro derivatives to FLT than that of 3'-azido derivatives to AZT. The *in vitro* hydrolysis rate data in Tables 3.17.1 and 3.17.2 confirmed this conclusion. However **91a** and **93** were less active than the corresponding 3'-azido derivatives **83d** and **87**, respectively. Other factors such as uptake rate into cells may contribute to the large variation in anti-HIV activities for these analogs. A comparative study to determine the *in vitro* stability of these analogs in culture medium is required to attain more accurate information that would be of value to explain the data.

#### 3.3.2.1.1.2.2.1. Cytotoxicity of 5'-*O*-myristoyl analogue derivatives of FLT

All analogs (**91a-93**) demonstrated lower toxicity ( $IC_{50} = 29-120$   $\mu$ M) than FLT ( $IC_{50} = 2.4$   $\mu$ M) (Table 3.12, Figure 3.4) in the CEM cell line. Compounds **91a-93** also displayed a 2.8-35.4 higher selectivity than FLT. The lower toxicity for 5'-*O*-ester prodrugs (**91a-93**) may be due to a sustained release of FLT upon the hydrolysis of the prodrugs. In addition, intracellular release of myristic acid analogs with toxicity profiles that differ from FLT may contribute to decreased cytotoxicity.

Most of the analogs (**91a-91c**, **92**, **93**) showed a lower toxicity in the 2.2.15 cell culture used for the anti-HBV assay (Table 3.13) than in the CEM-SS cell line (Table 3.12). For example, **91a** showed  $IC_{50}$  values of 29 and 417  $\mu$ M in CEM-SS cells and 2.2.15 cell cultures, respectively.

#### 3.3.2.1.2. Anti-HBV activity of 5'-*O*-myristoyl analogue derivatives of AZT and FLT

AZT fails to suppress the replication of HBV in patients with HIV and HBV infection and it is not a very effective against HBV replication in 2.2.15 cells even at the highest

possible concentration (20  $\mu\text{M}$ ) that did not affect cellular growth (Aoki-Sei *et al.*, 1991, Korba *et al.*, 1991). AZT had no appreciable effects on HBV DNA in HIV patients with chronic hepatitis B, despite long term therapy (Marcellin *et al.*, 1989) and it exhibited no inhibitory activity against HBV DNA polymerase in *in vitro* assays (Nordenfelt *et al.*, 1987). Since AZT is metabolized in the liver, effective levels of the active triphosphate may not be present in hepatocytes. However, it was reported that AZT-TP is a potent inhibitor of HIV reverse transcriptase (Matthes *et al.*, 1987) and that it also inhibits HBV DNA polymerase activity *in vitro* ( $\text{EC}_{50} = 0.15 \mu\text{M}$ ) (Meisel *et al.*, 1990). Zidovudine activity against hepatitis B virus appears to be related to a reverse transcription phase in the replication of single-stranded viral DNA, carried out by viral DNA polymerase (Summers *et al.*, 1982).

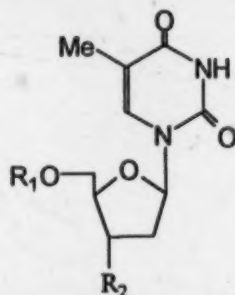
FLT is one of the most effective antiviral agents. At a concentration of 0.03  $\mu\text{M}$ , a greater than 90 % reduction in HBV DNA synthesis occurs. FLT was found to be a strong competitive inhibitor of HBV DNA polymerase with an inhibition constant ( $K_i$ ) of 0.04  $\mu\text{M}$  (Eriksson *et al.*, 1988, Matthes *et al.*, 1992). FLT 5'-triphosphate is also a potent inhibitor of endogenous hepatitis B virus (HBV) DNA polymerase and the production of HBV in HepG2 cells transfected with HBV DNA *in vitro* (Matthes *et al.*, 1988). FLT-TP inhibits HBV DNA polymerase by 50% at 0.15  $\mu\text{M}$  (Meisel *et al.*, 1990) and by 88% at 1  $\mu\text{M}$  (Eriksson *et al.*, 1988, Matthes *et al.*, 1992).

Dideoxycytidine (ddC) inhibits the replication of hepatitis B virus (HBV), but its clinical use is limited by peripheral neuropathy (Yarchoan *et al.*, 1988).

To treat hepatitis B more effectively, it would be desirable to direct a larger fraction of administered antiviral nucleosides to the liver using a prodrug. Many nucleosides active against HBV are known to be very toxic to nonhepatic tissues *in vivo* which may result in inadequate levels in liver tissue. Prodrugs targeted to liver might be expected to reduce drug toxicity and improve efficacy, if the liver itself is not the target for toxicity. Several phospholipid prodrugs of anti-HBV nucleosides like ddC have been synthesized for this purpose previously. Dioleoylphosphatidyl-ddC was less effective *in vitro* than ddC. Its toxicity in 2.2.15 cells was only 20% that of free ddC (5) and its *in vitro* selectivity index was 56 vs 30 for ddC. Intraperitoneal administration of a liposomal formulation of this

prodrug to mice resulted in a 40-fold increased retention relative to ddC in the disease target (liver) without a significant increase in the toxicity target (sciatic nerve) (Hostetler *et al.*, 1994).

**Table 3.13.** Relative *in vitro* potency and selectivity of 5'-O-myristoyl analogue derivatives of FLT and AZT as inhibitors of HBV DNA replication in 2.2.15 cell culture (HBV virion extracellular DNA).



| No.              | R <sub>1</sub>                                      | R <sub>2</sub> | IC <sub>50</sub><br>(μM) <sup>a</sup> | EC <sub>50</sub><br>(μM) <sup>b</sup> | EC <sub>90</sub><br>(μM) <sup>c</sup> | SI <sup>d</sup> |
|------------------|-----------------------------------------------------|----------------|---------------------------------------|---------------------------------------|---------------------------------------|-----------------|
| 66e              | MeO(CH <sub>2</sub> ) <sub>11</sub> COOH            | —              | 379±22                                | 22±1.8                                | 60±6.3                                | 6.3             |
| 83e              | MeO(CH <sub>2</sub> ) <sub>11</sub> CO              | N <sub>3</sub> | 727±19                                | 2.7±0.3                               | 17±1.8                                | 43              |
| 91a              | Br(CH <sub>2</sub> ) <sub>11</sub> CO               | F              | 417±38                                | 8.0±1                                 | 48±7.8                                | 8.7             |
| 91b              | MeO(CH <sub>2</sub> ) <sub>11</sub> CO              | F              | 186±20                                | 2.8±0.3                               | 8.1±1.2                               | 23              |
| 91c              | EtS(CH <sub>2</sub> ) <sub>11</sub> CO              | F              | 193±14                                | 21±2                                  | 50±5                                  | 3.9             |
| 91d              | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO  | F              | 61±6                                  | 6.1±1.4                               | 18±2                                  | 3.4             |
| 92               | EtS(CH <sub>2</sub> ) <sub>10</sub> CO              | F              | 275±23                                | 22±2                                  | 51±6                                  | 5.4             |
| 93               | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO | F              | 384±25                                | >30                                   | >30                                   | ND              |
| FLT <sup>e</sup> | H                                                   | F              | 361 ± 24                              |                                       | 40 ± 4                                | 9               |
| ddC <sup>f</sup> |                                                     |                | 252±28                                | 1.3±0.1                               | 6.8±0.7                               | 37              |
| 3TC <sup>g</sup> |                                                     |                | 1180±125                              | 0.033±0.004                           | 0.188±0.020                           | 6280            |

<sup>a</sup> IC<sub>50</sub> = 50% inhibitory concentration (±SD). <sup>b</sup> EC<sub>50</sub> = 50% effective concentration (±SD). <sup>c</sup>

EC<sub>90</sub> = 90% effective concentration (±SD). <sup>d</sup> Selectivity Index (S.I.: IC<sub>50</sub>/EC<sub>90</sub>). <sup>e</sup> Data for FLT

taken from Korba *et al.*, 1992 against HBV DNA synthesis. <sup>f</sup> ddC = 2',3'-dideoxycytidine. <sup>g</sup> Data for 2',3'-dideoxy-3'-thiacytidine taken from Xie *et al.*, 1995.

Myristoyl derivatives of FLT and AZT, that are potent *in vitro* inhibitors of HBV



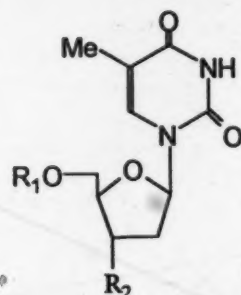
production in a cellular system (2.2.15 cells) were synthesized to increase nucleoside uptake by the liver, to reduce tissue toxicity and to increase their accumulation in hepatocytes. Despite distinct differences between HBV and HIV infections, HBV is a potential cofactor for HIV infection. Since multiple concomitant viral infections frequently occur, there would be a potential clinical benefit to treat both HBV and HIV infections with a single active compound. As mentioned earlier, all of the myristoyl analog prodrugs showed a high *in vitro* anti-HIV activity.

These prodrug esters are thought to be effective against hepatitis B virus due to their ability to inhibit a reverse transcriptase that the virus utilizes at some stage of its life cycle after hydrolysis to FLT or AZT. The ability of these prodrugs to FLT or AZT to inhibit hepatitis B virus (HBV) replication in chronically HBV-producing human liver cells (2.2.15 cells) was determined. The anti-HBV activity for selected analogs is summarized in Tables 3.13, 3.14 and Figure 3.5. Some of the important results are discussed in the section that follows:

(1)-3'-Azido-2',3'-dideoxy-5'-O-(12-methoxydodecanoyl)thymidine (83e) exhibited comparable potency to 2',3'-dideoxycytidine, but it was significantly more selective. AZT is one of the least effective nucleosides against HBV replication in 2.2.15 cells, but the 5'-O-(12-methoxydodecanoyl) ester (83e) showed high activity ( $EC_{50} = 2.7 \pm 0.3 \mu M$ ) and low toxicity ( $IC_{50} = 727 \pm 19 \mu M$ ) (Table 3.13). The observation that 12-Meo-AZT (83e) also inhibits HBV replication, a property not exhibited by AZT, was unexpected. The precise basis for this broader spectrum of activity of an antiretroviral agent to include HBV is unknown, but it could be due to an increased concentration of AZT inside infected cells. In a similar unexpected observation reported by Horrobin *et al.* (1993), gamma linoleic acid-AZT (GLA-AZT) also inhibited herpes virus replication, a property not found with either GLA or AZT individually. The precise basis for the spectrum of activity of an anti-retroviral agent to include the herpes viruses is unknown but could be due to the enhanced permeation of the prodrug ester through cell membranes (Horrobin *et al.*, 1993). While the anti-HBV activity of 83e was lower than ddC *in vitro*, it was at least 2.5-fold less cytotoxic than ddC. The selectivity index for ddC was 37 vs 43 for 83e (HBV RI extracellular DNA) and 19 vs 27 for 83e in 2.2.15 cell culture (HBV RI

intracellular DNA). The reasons for the improved selectivity of 83e, which are not currently clear, will require studies to determine 2.2.15 cell uptake and metabolism.

**Table 3.14.** Relative *in vitro* potency and selectivity of 5'-*O*-myristoyl analogue derivatives of FLT and AZT as inhibitors of HBV DNA replication in 2.2.15 cell culture (HBV RJ intracellular DNA).



| No.              | R <sub>1</sub>                                      | R <sub>2</sub> | IC <sub>50</sub><br>(μM) <sup>a</sup> | EC <sub>50</sub><br>(μM) <sup>b</sup> | EC <sub>90</sub><br>(μM) <sup>c</sup> | SI <sup>d</sup> |
|------------------|-----------------------------------------------------|----------------|---------------------------------------|---------------------------------------|---------------------------------------|-----------------|
| 66e              | MeO(CH <sub>2</sub> ) <sub>11</sub> COOH            | —              | 379±22                                | 31±3.4                                | 90±11                                 | 4.2             |
| 83e              | MeO(CH <sub>2</sub> ) <sub>11</sub> CO              | N <sub>3</sub> | 727±19                                | 4.9±0.5                               | 27±2.9                                | 27              |
| 91a              | Br(CH <sub>2</sub> ) <sub>11</sub> CO               | F              | 417±38                                | 19±2.1                                | 78±7.8                                | 5.3             |
| 91b              | MeO(CH <sub>2</sub> ) <sub>11</sub> CO              | F              | 186±20                                | 5.6±0.6                               | 17±1                                  | 11              |
| 91c              | EtS(CH <sub>2</sub> ) <sub>11</sub> CO              | F              | 193±14                                | 34±4                                  | 86±8                                  | 2.2             |
| 91d              | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO  | F              | 61±6                                  | 11±2                                  | 28±3                                  | 2.2             |
| 92               | EtS(CH <sub>2</sub> ) <sub>10</sub> CO              | F              | 275±23                                | 33±4                                  | 70±8                                  | 3.9             |
| 93 <sup>e</sup>  | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO | F              | 384±25                                | >30                                   | >30                                   | ND              |
| FLT <sup>e</sup> | H                                                   | H              | 361 ± 24                              |                                       | 40 ± 4                                | 9               |
| ddC <sup>f</sup> |                                                     |                | 252±28                                | 2.6±0.3                               | 13±1.8                                | 19              |
| 3TC <sup>g</sup> |                                                     |                | 1180                                  | 0.076±0.008                           | 0.293±0.03                            | 4030            |

<sup>a</sup> IC<sub>50</sub> = 50% inhibitory concentration. <sup>b</sup> EC<sub>50</sub> = 50% effective concentration (±SD). <sup>c</sup> EC<sub>90</sub> = 90% effective concentration (±SD). <sup>d</sup> Selectivity Index (S.I.: IC<sub>50</sub>/EC<sub>90</sub>). <sup>e</sup> Data for FLT taken from Korba *et al.*, 1992 against HBV DNA synthesis. <sup>f</sup> ddC = 2',3'-dideoxycytidine. <sup>g</sup> Data for 2',3'-dideoxy-3'-thiacytidine taken from Xie *et al.*, 1995.

(2) 3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-methoxydodecanoyl) ester (91b) exhibited the

highest activity ( $EC_{50} = 5.6 \mu M$ ) among the 3'-fluoro analogs tested (91a-93), however it was less active and more cytotoxic than 83e, probably due to differences in partition coefficient and cellular uptake (Tables 3.13, 3.14, Figure 3.5).

(3) 12-Methoxydodecanoic acid (66e) exhibits potent activity and is less toxic than ddC (5). This demonstrates the importance of this fatty acid with respect to the activity for 83e and 91b which were the most potent analogs tested (Tables 3.13, 3.14).

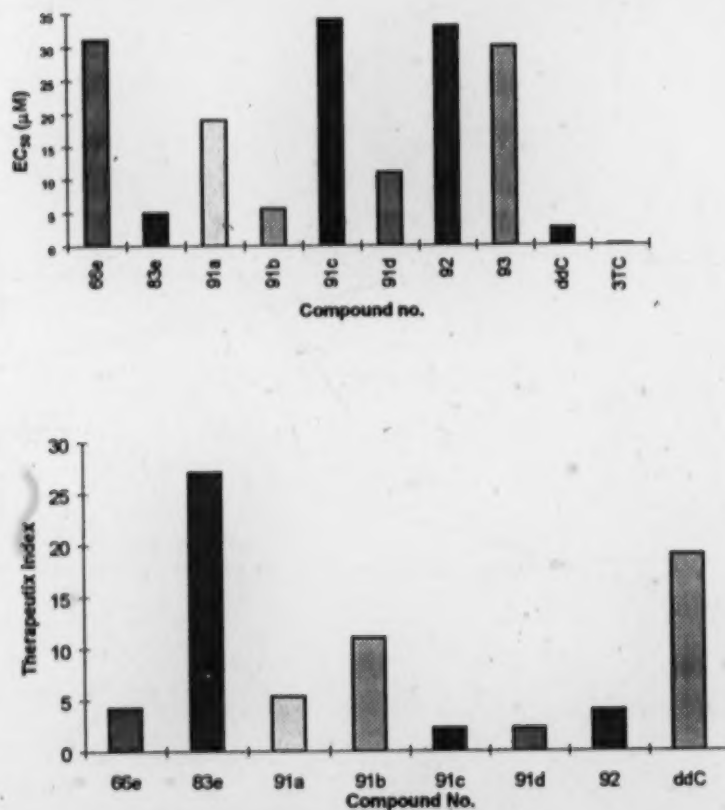


**Figure 3.5.** Comparative potency and selectivity of 5'-O-myristoyl analogue derivatives of AZT and FLT as inhibitors of HBV DNA replication in 2.2.15 cell culture (HBV RI intracellular DNA).

These data indicate that parenteral administration of myristoyl analog ester prodrugs of antiviral nucleosides may increase *in vivo* efficacy and selectivity of nucleoside therapy for hepatitis B infection. One could conclude that myristoyl analog ester prodrugs of

antiviral nucleosides may represent a novel approach to improving efficacy and reducing the toxicity of antiviral agents used in treating HBV infections. Further studies in animal models of hepatitis B will be needed to evaluate this possibility.

A group of myristoyl analog ester prodrugs was also evaluated against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2). At a test compound concentration of 10 µg/mL, none of these esters significantly inhibited the replication of HBV-infected hepatocytes. Viral replication (the values are the average of two different experiments) was estimated from the amount of HBV DNA present in the supernatant of the cell culture after incubation with the test compound (10 µg/mL). The data are expressed as a percentage of control values where the cell culture was not incubated with the test compound. The % of viral replication for selected compounds [compound number (% of viral replication)] was 82c (104.2%); 82d (110.1%); 82e (104.6%); 83a (70.4%); 83c (86.9%); 83d (57.6%); 83e (75.8%); 83f (80.6%); 83h (59.7%); 84a (140.5%); 84b (102.6%); 84d (51.5%); 85a (102.9%); 85b (65.23%); 86(69.3%); 87 (44.8%); 91a (57.6%); 91b (91.2%); 91c (88.1%); 91d (126.7%); 92 (87.7%), 93 (85.4%); AZT (40.81%); FLT (10.8%); 3TC (0.0%) and control (100.0%). The activity of 83e and 91a-93 in these two assays are different, since the harvesting time, the duration of exposure, and the time at which cells were exposed to the test compound, differ (See section 3.3.1.1.2.3).

### 3.3.2.2. Physicochemical properties of 5'-O-myristoyl analogue derivatives of AZT and FLT

#### 3.3.2.2.1. Physical appearance

Many prodrugs of AZT and FLT were isolated as oils, that is probably due to loss of the tightly packed crystal structure for AZT and FLT and the increased steric bulk introduced after esterification of the free nucleoside 5'-hydroxyl group and the loss of intermolecular hydrogen bonding between the 3'- and 5'-hydroxyl groups. In contrast, the 5'-O-myristoyl analogue derivatives of thymidine, which are solids, can still undergo intermolecular hydrogen bonding via the 3'-hydroxyl group.



### 3.3.2.2.2. Partition coefficient and HPLC retention time determination, and their correlations for 5'-O-myristoyl analogue derivatives of AZT, FLT and dThd

One of the primary objectives of this project was to design and synthesize prodrugs of AZT with improved permeability properties in order to achieve a higher concentration of the prodrug in the central nervous system (CNS) and in the HIV and HBV target cells such as liver cells and macrophages. In the absence of an active nucleoside transport system, the enhanced lipophilicity of these analogs, relative to parent nucleoside, should increase their ability to cross plasma membrane and the BBB by diffusion. This postulate is based on the observation that increasing the lipophilicity of compounds with a molecular weight of less than 400 has been reported to improve brain permeability (Kumar *et al.*, 1994b). In order to predict passive diffusion through cell membranes, the partition coefficients of some 5'-O-myristoyl analogs of AZT, FLT and dThd in a *n*-octanol/phosphate buffer (0.2 M, pH 7.4) system at room temperature were determined. Among these parent nucleosides dThd, AZT and FLT, AZT is the most lipophilic compound due to the -N<sub>3</sub> at the 3'-position of the sugar moiety. This is consistent with the fact that the azido substituent possesses very different physicochemical properties compared to a hydroxy group, especially steric size and lipophilic ( $\pi$ -value) effects (Hansch *et al.*, 1973).

Esterification of the C-5' hydroxyl group of dThd, AZT and FLT resulted in an increased lipophilicity. The partition coefficient, which is an indicator of the ability of a compound to cross the BBB, for these analogs were all larger (Log P = 3.96-8.14 range) than that for dThd (Log P = -1.55), FLT (Log P = -0.52) and AZT (Log P = 0.06), respectively.

Experimental partition coefficients (Log P) for the 5'-O-myristoyl analogue derivatives of thymidine (81a-h) were determined using a mixture of *n*-octanol and phosphate-buffered solution, pH 7.4, at room temperature (see Table 3.15.1). Log P values were also estimated using the PALLAS computational program, PrologP 5.1, to calculate theoretical Log P values for 5'-O-esters of thymidine in an *n*-octanol/water system. Experimental partition (Log P = 4.15 to >5) coefficients, which were calculated for these esters (81a-h)



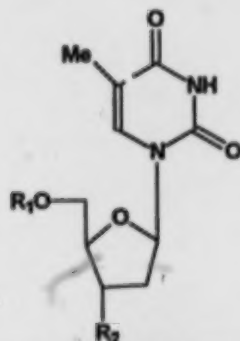
investigated (Table 3.15.1), extended over a broad range (Parang *et al.*, 1996).

Due to the very high lipophilicity of most ester analogs of AZT, FLT and dThd the absolute measurement of their P value was not feasible, and the values for the long chain esters were too high to be determined. These results are in agreement with the data of Kawaguchi *et al.*, (1990). Therefore, it was not possible to measure the exact P value for most ester prodrugs since no change in ester concentration in *n*-octanol was detected during partition coefficient determination. With very lipophilic molecules, for example, it is evident that relatively small volumes of nonpolar solvent must be used or there will be insufficient material left in the aqueous phase for analysis. For example, if a solute has a P value of 200, and 20 mg was partitioned between equal volumes, the aqueous phase would end up with only 0.1 mg. If the analytical procedure has an inherent error of 0.05 mg/100 mL, the P value could vary between 133 and 400. If, however, 200 mL of water and 5 mL of nonpolar solvent were used, the water layer would contain 3.5 mg or 1.75 mg/100mL and the same analytical accuracy would limit the range of P values from 194 to 206. With good analytical procedures and proper volume choices of solvent, Log P values in the range -5 to +5 can be measured (Leo *et al.*, 1971).

Due to the high lipophilicity of the ester prodrugs studied, the determination of experimental partition coefficient determination was very difficult. Small changes in the concentration of the ester in the *n*-octanol phase (5 mL) was measurable if a higher volume of buffer phase (10 mL) for compounds with Log P < 5. In most cases an approximate value was reported. To get a better comparison between different esters, the HPLC retention time and calculated Log P values acquired using the PALLAS program were used (Tables 3.15.1 and 3.15.2).

The estimated partition coefficient of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) was highest in this series. Increasing the length of the ester chain to 14 atoms or introducing lipophilic substituents (bromine, phenyl, phenoxy) affected lipophilicity dramatically.

Table 3.15.1. Physical properties of 5'-*O*-myristoyl analogue derivatives of dThd and AZT (81a-83i).

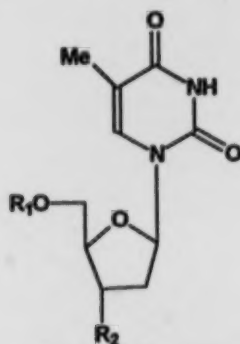


| No. | R <sub>1</sub>                                                                      | R <sub>2</sub> | RT<br>(min) <sup>a</sup> | Log P<br>(exp.) <sup>b</sup> | Log P<br>(calcd) <sup>c</sup> | Log D <sub>7.4</sub><br>(calcd) <sup>d</sup> |
|-----|-------------------------------------------------------------------------------------|----------------|--------------------------|------------------------------|-------------------------------|----------------------------------------------|
| 81a | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                                 | OH             | 48.3                     | > 5                          | 5.57                          | 5.57                                         |
| 81b | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO                           | OH             | 79.8                     | > 5                          | 6.53                          | 6.38                                         |
| 81c | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                               | OH             | 14.7                     | 4.30                         | 4.11                          | 4.22                                         |
| 81d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | OH             | 20.6                     | 4.79                         | 4.62                          | 4.73                                         |
| 81e | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO                | OH             | 20.6                     | 4.71                         | 4.55                          | 4.34                                         |
| 81f | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(N <sub>3</sub> )CO              | OH             | 54.7                     | > 5                          | 6.38                          | 6.38                                         |
| 81g | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                                  | OH             | 11.2                     | 4.15                         | 3.96                          | 3.95                                         |
| 81h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | OH             | 17.2                     | 4.64                         | 4.48                          | 4.48                                         |
| 82a | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO               | N <sub>3</sub> | 12.0                     | 4.30                         | 4.51                          | 4.60                                         |
| 82b | N <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO  | N <sub>3</sub> | 11.9                     | 4.80                         | 4.57                          | 4.60                                         |
| 82c | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO | N <sub>3</sub> | 31.1                     | > 5                          | 5.67                          | 5.70                                         |
| 82d | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> O(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 19.8                     | > 5                          | 5.67                          | 5.70                                         |
| 82e | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> S(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 34.2                     | > 5                          | 6.16                          | 6.20                                         |
| 83a | F(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 20.5                     | > 5                          | 5.51                          | 5.50                                         |
| 83b | I(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 45.7                     | > 5                          | 6.55                          | 6.60                                         |
| 83c | Cl(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 29.8                     | > 5                          | 6.03                          | 6.00                                         |
| 83d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 35.1                     | > 5                          | 6.24                          | 6.20                                         |
| 83e | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | N <sub>3</sub> | 20.5                     | > 5                          | 5.09                          | 5.10                                         |
| 83f | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO                | N <sub>3</sub> | 50.1                     | > 5                          | 6.68                          | 6.70                                         |
| 83g | Ph(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 92.8                     | > 5                          | 7.81                          | 7.80                                         |
| 83h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | N <sub>3</sub> | 25.6                     | > 5                          | 6.09                          | 6.10                                         |
| 83i | PhO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | N <sub>3</sub> | 61.06                    | > 5                          | 7.36                          | 7.40                                         |

(a) Retention time: Methanol/Water (75/25, v/v), flow rate of 1 mL/min using reverse phase HPLC. (b) Partition coefficient in *n*-octanol and phosphate buffer at pH 7.4 (0.2 M).

(c) Partition coefficient of the ester analog calculated using the Prolog 5.1 program. (d) Distribution coefficient of the fatty acid at pH 7.4 calculated using the PrologD 2.0 prediction program.

Table 3.15.2. Physical properties of 5'-*O*-myristoyl analogue derivatives of AZT (84a-87) and FLT (91a-93).



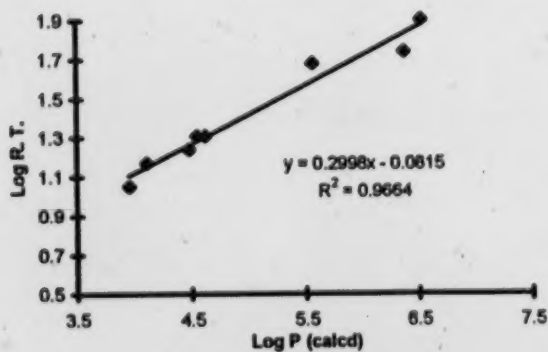
| No.  | R <sub>1</sub>                                                       | R <sub>2</sub> | RT<br>(min) <sup>a</sup> | Log P<br>(exp.) <sup>b</sup> | Log P<br>(calcd) <sup>c</sup> | Log D <sub>7.4</sub><br>(calcd) <sup>d</sup> |
|------|----------------------------------------------------------------------|----------------|--------------------------|------------------------------|-------------------------------|----------------------------------------------|
| 84a  | MeO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> | 14.9                     | 4.80                         | 4.57                          | 4.60                                         |
| 84b  | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | N <sub>3</sub> | 33.8                     | > 5                          | 6.16                          | 6.20                                         |
| 84c  | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                | N <sub>3</sub> | 24.6                     | > 5                          | 5.72                          | 5.70                                         |
| 84d  | 4-I-PhO(CH <sub>2</sub> ) <sub>10</sub> CO                           | N <sub>3</sub> | 93.7                     | > 5                          | 8.10                          | 8.10                                         |
| 84e  | PhO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> | 41.7                     | > 5                          | 6.84                          | 6.80                                         |
| 84f  | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                   | N <sub>3</sub> | 20.2                     | > 5                          | 5.57                          | 5.60                                         |
| 85a  | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(F)CO             | N <sub>3</sub> | 74.3                     | > 5                          | 7.41                          | 7.40                                         |
| 85b  | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO            | N <sub>3</sub> | 133.19                   | > 5                          | 8.14                          | 8.10                                         |
| 86   | Ph(CH <sub>2</sub> ) <sub>9</sub> CO                                 | N <sub>3</sub> | 41.4                     | > 5                          | 6.77                          | 6.80                                         |
| 87   | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | N <sub>3</sub> | 118.7                    | > 5                          | 7.19                          | 7.20                                         |
| 91a  | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                | F              | 27.3                     | > 5                          | 5.65                          | 5.70                                         |
| 91b  | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                               | F              | 16.0                     | 4.30                         | 4.50                          | 4.50                                         |
| 91c  | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO | F              | 36.6                     | > 5                          | 6.09                          | 6.10                                         |
| 91d  | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                   | F              | 21.8                     | > 5                          | 5.51                          | 5.50                                         |
| 92   | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | F              | 25.3                     | > 5                          | 5.51                          | 5.60                                         |
| 93   | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | F              | 72.4                     | > 5                          | 6.60                          | 6.60                                         |
| AZT  | H                                                                    | N <sub>3</sub> | 4.3                      | 0.11                         | 0.06                          | 0.06                                         |
| FLT  | H                                                                    | F              | 4.1                      | -0.30                        | -0.52                         | -0.53                                        |
| dThd | H                                                                    | OH             | 3.8                      | -1.19                        | -1.55                         | -1.25                                        |

(a) Retention time: Methanol/Water (75/25, v/v), flow rate of 1 mL/min using reverse phase HPLC. (b) Partition coefficient in *n*-octanol and phosphate buffer at pH 7.4 (0.2 M).

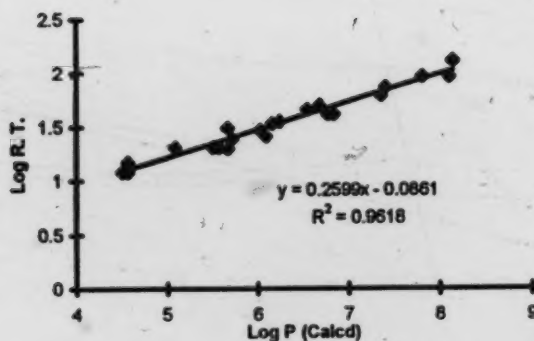
(c) Partition coefficient of the ester analog calculated using the Prolog 5.1 program. (d) Distribution coefficient of the fatty acid at pH 7.4 calculated using the PrologD 2.0 prediction program.

The relative lipophilicities of these compounds can also be determined from their retention times on reverse-phase HPLC chromatography where more polar compounds, possess a shorter retention time. Balzarini *et al.*, (1989) reported that there was a correlation (correlation coefficient  $> 0.97$ ) between P and HPLC retention time values for 36 anti-HIV nucleoside analogs that were investigated. Their results suggest that there are close linear correlations between Log P and retention time values for 2',3'-dideoxynucleosides, purine 2',3'-dideoxynucleosides, and 5-halogeno-substituted 3'-fluoro-2',3'-dideoxynucleosides. Correlations between Log P and Log retention time for AZT, FLT, and dThd derivatives also show a linear relationship that is illustrated in Figure 3.6. The correlation coefficient ( $R^2$ ) for this relationship (0.89-0.96) may provide a useful tool to predict the partition coefficients of other ester analogs from their retention time using the same HPLC conditions.

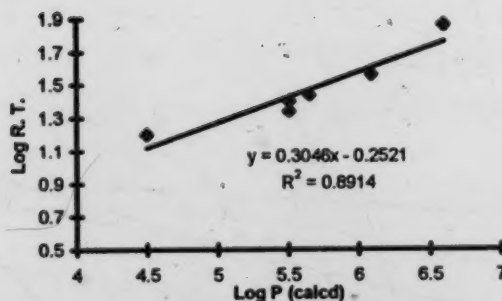
All of the 3'-fluoro derivatives (91a-93) showed a shorter retention time and lower partition coefficient than the corresponding 3'-azido derivatives (83d-f, 83h, 84b, 87) since FLT is less lipophilic than AZT. The lower lipophilicity for 5'-*O*-myristoyl analogs of dThd, relative to those for AZT and FLT, is due to the presence of the 3'-hydroxy group in thymidine derivatives.



5'-*O*-Esters of dThd



5'-O-Esters of AZT



5'-O-Esters of FLT

**Figure 3.6.** The correlation between Log retention time and lipophilicity (Log P) for 5'-O-esters of dThd, FLT and AZT.

#### 3.3.2.2.2.1. Partition coefficient and anti-HIV activity correlations for 5'-O-myristoyl analogue derivatives of AZT, FLT and dThd

According to Hansch and Fujita (Hansch *et al.*, 1964), there should be an optimum Log P for achieving the best biological activity.

There does appear to be a strong correlation between anti-HIV activity and Log P for 5'-O-myristoyl analogue derivatives of thymidine (81a-81h), since the contribution of partition coefficient in the QSAR regression equation confirms the importance of this descriptor with respect to anti-HIV-1 activity exhibited by these esters (81a-h). Only those compounds (81d, 81e and 81h) with an experimental partition coefficient (Log P) in



the 4.6-4.8 range showed medium *in vitro* anti-HIV activity. However, additional studies using more compounds are required to make a conclusion regarding a direct correlation between anti-HIV activity and Log P values. The contribution of partition coefficient to the QSAR regression equation confirms the importance of this descriptor with respect to anti-HIV-1 activity for these esters (81a-h) (see section 3.3.2.1.1.3.3).

No relationship was found between optimum selectivity and potency with Log P values for 5'-O-fatty acyl derivatives of AZT and FLT. Although no general trends were evident for this relationship, highly lipophilic compounds (83f, 83g, 83i, 84d, 84e, 85a, 85b, 86) (Log P > 6.6) showed lower anti-HIV activity than other analogs. Compounds with a high partition coefficient probably undergo less cellular uptake into cells. For 5'-O-myristoyl analogue derivatives of AZT, optimum selectivity and potency was generally observed mostly for analogs (82c, 82e, 83d) with partition coefficients in the range 5.67-6.24. However, some compounds (82d, 83c, 83h, 84b, 84c) which had Log P values in this range exhibited lower activity against HIV-1. Accordingly, there appear to be several factors which determine apparent *in vitro* anti-HIV activity of each analog that may include partition coefficient, rate of uptake and intracellular rate of hydrolysis of the 5'-O-ester.

### 3.3.2.3. Enzymatic and chemical hydrolysis of 5'-O-myristoyl analogue derivatives of AZT, FLT and dThd

#### 3.3.2.3.1. Esterases

Esterases are enzymes that hydrolyze esters. This is a broad definition and includes many enzymes which are not the concern of this thesis. One of the main reasons for the incomplete understanding of the properties of the many esterases that exist in tissues is their overlapping substrate specificity and the difficulty in determining the action of individual esterases. One substrate may be hydrolyzed by several enzymes. Hydrolases that only cleave ester bonds are called carboxylic ester hydrolases (carboxyesterases) (EC 3.1.1). Although carboxyesterases is a collective term, the related enzymes can be classified into various groups which possess different substrate specificity. Many carboxyesterases have a serine residue in their active site and have a preference for

lipophilic substrates relative to polar and charged substrates. Since individual esterases have different specificities, each substrate will present a different capacity to undergo cleavage when subtle structural changes are involved (Valette *et al.*, 1996, Heymann, 1980, Satoh, 1987).

Pig liver carboxyesterase is a mixture of very similar, but chemically different enzymes with differing substrate specificities. The pig liver esterase (carboxyl esterase hydrolase, EC 3.1.1.1) is a highly active serine hydrolase that contains two active sites in each molecule (MW 168 KD). Two sites have been proposed that include an esterolytic site and a modifier site. The control or binding site has been shown to be hydrophobic but the esterolytic site is a hydrolytic site. The binding of substrate to the binding site accelerates the reaction in the esterolytic site (Ocken *et al.*, 1970). Depending on the enzyme's catalytic properties, it can be classified as a cholinesterase enzyme (Junge *et al.*, 1979). Acetylcholinesterase, the enzyme found in nervous tissues of all species of animals, catalyzes the hydrolysis of the neurotransmitter acetylcholine after it has served its neurohumoral transmission function.

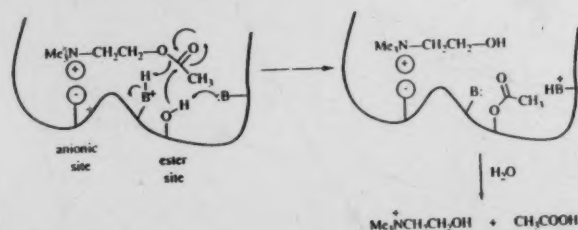


Figure 3.7. Acetylcholinesterase-catalyzed hydrolysis of acetylcholine (Silverman *et al.*, 1992).

The active site of the acetylcholinesterase is believed to contain two important binding sites, the anionic site and ester site. Catalytic hydrolysis of the acetoxy group occurs at the esterolytic site (Silverman *et al.*, 1992) (Figure 3.7).

#### 3.3.2.3.2. Stability of prodrug esters toward esterases

Each prodrug will be activated (ester cleavage) according to its relative overall substrate capacity towards carboxyesterases, that will determine the rate of the activation step. To be useful, the ester prodrug concept requires the ester to be stable in extracellular

media but to undergo ester cleavage after uptake in cells. Such a strategy presumes important differences in the carboxyesterase content of extra- and intracellular media and/or enzymes. The usefulness of the prodrugs to AZT will depend not only on the stability of the prodrug prior to its passage across the cell membrane and into the CNS, but also upon its intracellular bioconversion to the parent compound, especially in the virally infected cells.

The stability of 5'-O-esters of AZT, FLT and dThd was determined following *in vitro* incubation in several media including porcine liver esterase, rat plasma and rat brain homogenate at 37 °C. The results of this study are summarized in Tables 3.16.1 and 3.16.2.

There are several reasons why prodrug esters of AZT and FLT were selected for this study. First, esterases are ubiquitous, and several types are present in blood, liver and other organs or tissues. Therefore, *in vitro* metabolic regeneration of the parent drug should occur readily. In addition, by selective esterification of AZT, FLT and dThd, it is possible to design derivatives with almost any desired hydrophilicity, lipophilicity, or *in vivo* lability with the latter property being dependent upon electronic and steric factors. Finally, esters showing a range of stabilities can be designed by appropriate manipulation of electronic and steric factors which will influence ester stability and/or hydrolysis rate. Therefore, a multitude of ester prodrugs can be prepared to address a wide variety of problems that can be circumvented using the prodrug approach (Silverman *et al.*, 1992).

The extent of ester hydrolysis was assessed by quantitating their hydrolysis to the acid and nucleoside moiety. The decrease in prodrug ester concentration was accompanied by an increase in the appearance of free parent nucleoside (AZT, FLT, dThd), demonstrating the presence of esterase or esterase-like activity under these conditions. For all enzyme preparations utilized, the decrease in concentration of the ester prodrug followed pseudo-first-order kinetics ( $C = C_0 e^{-kt}$ , where  $C_0$  represents the initial concentration of ester prodrug, and  $C$  is the concentration of ester at time  $t$ ). Figure 3.8 shows the time course for the disappearance of 85b in a solution of porcine liver esterase.

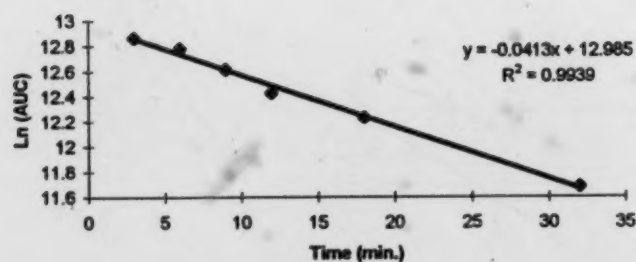


**Figure 3.8.** Semi-logarithmic plot of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) peak area vs time upon incubation with porcine liver esterase.

#### 3.3.2.3.2.1. Important factors determining the rate of enzymatic hydrolysis of prodrug esters

The rate of regeneration of the active(s) drug from the prodrug is dependent upon the rate of hydrolysis of the ester prodrug by esterases. However, the specificity of various esterases toward substrate variation is poorly understood. The susceptibility of prodrugs to rapid degradation by plasma esterases constitutes a potential weakness of the prodrug approach. Appropriate structural modifications of esters can render them more resistant to degradation by plasma enzymes. There are different factors involved in the stability of these esters. Differences in their rates of ester hydrolysis is dependent upon the nature, lipophilicity, and steric size of the acyl group, the nature of the nucleoside moiety and the type or concentration of the esterase in the medium.

##### 3.3.2.3.2.1.1. Steric effects

The bioconversion kinetics of ester prodrugs to the parent drug(s) appears to dependent upon the steric size of the acyl substituent. More sterically hindered esters undergo slower hydrolysis. Increasing the length of the fatty acid side chain length decreased cleavage of the ester bond. Binding of the substrate to the hydrophobic pocket at the active site of carboxyesterase is involved in this interaction. Large groups at the



terminal end of the fatty acid chain decreased hydrolysis of the ester. This is attributed to the steric hindrance of the substituent, thus inhibiting its binding to the active center of the enzyme. Therefore, the substituent steric effects play an important role in determining the rate and/or extent of enzymatic cleavage of the ester prodrugs by the esterase. The 2-bromo analog (85b) is more stable toward plasma esterase than the 2-fluoromystoyl analog (85a) due to the longer steric size of bromine at the  $\alpha$ -position, which partially blocks binding to the esterolytic site of the enzyme with the ester group. The *in vitro* kinetics in biological fluids indicated slow conversion of the 2-bromo derivative (85b) to AZT in all media. This increased stability can be attributed to the close proximity of the bromine group. The smaller size of  $\alpha$ -F substituent present in 85a, makes it more sensitive to the nucleophilic attack by the esterolytic site of the enzyme.

The relationship between increased linear chain length of the substrate and decreased enzymatic hydrolysis might be attributed to the possible enhanced steric hindrance between the substrate and a hydrophobic pocket at the active center of ester hydrolases, a phenomenon which may be responsible for the higher stability of ester prodrugs 83i, 84d, 84e, 85b, 87 and 93. An increase in the steric bulk of the 5'-acyl substituent tends to decrease the hydrolysis rate and appears to inhibit binding of the substrate to the active center of the enzyme. Among the group of aromatic esters, the phenoxy derivatives (83i, 84d, 84e) appeared to be much more stable than phenyl derivatives (83g, 86). Therefore, the rate of hydrolysis decreases with an increase in the acyl chain length and insertion of a bulky group like phenyl, and phenoxy at the terminal position.

According to Kawaguchi *et al.* (1985a, 1985b, 1990, 1992) the enzymatic hydrolysis rate of 5'-O-esters of AZT in the presence of different rat enzyme preparations, including plasma and brain homogenate, is dependent upon the length of the acyl chain. For ester prodrugs of AZT with normal acyl chains, a parabolic relationship between acyl chain length and susceptibility to enzymatic hydrolysis has been reported. That is, acyl chains with 8-10 carbons show the highest enzymatic cleavage. A decrease or increase in the acyl chain length decreases susceptibility to enzymatic hydrolysis. Similar results were obtained in current experiments, since the longer acyl chains present in 83i, 84d, 84e, 85b, 87 and 93 decreased the extent of hydrolysis in different media. The results presented in Tables



3.16.1 and 3.16.2, indicate that regeneration of AZT is dependent upon the nature of the substituent at the 5'-position. The % hydrolysis decreases with an increase in the chain length (more than 14 carbon). A larger % hydrolysis could be due to enhanced interaction between the substrate and the enzyme. However, an increase in the steric size of 5'-acyl substituents tends to decrease the % hydrolysis, demonstrating possible steric effects.

#### 3.3.2.3.2.1.2. Nucleoside effect

The 3'-fluoro derivatives showed a higher susceptibility to ester cleavage than 3'-azido derivatives (Table 3.16.2). For example, 12-methoxydodecanoyl ester derivative of FLT (91b) showed a higher susceptibility in all enzymic preparations than the corresponding ester derivative of AZT (83e) (Tables 3.17.1 and 3.17.2). This more extensive hydrolysis for the 3'-fluoro esters (91a-93) might be due to a possible electrostatic interaction between the substrate and the enzyme since the fluorine atom possess strong electron withdrawing properties and it is smaller compared to azide substituent. In addition, the interaction of these esters 91a-93 with esterase binding sites may be sterically more facile.

#### 3.3.2.3.2.1.3. Medium effect

The ester cleavage degradation half-life as significantly dependent upon the type or concentration of esterase in tissue or plasma concentration. The ester cleavage susceptibility of the prodrug varied with the ester promoiety. The 2-fluoro ester (85a) showed a high susceptibility to tissue homogenates and plasma, while 2-bromo (85b) was relatively stable in all enzymic preparations (Figures 3.9, 3.10). Most of the esters undergo rapid enzymatic hydrolysis to AZT or FLT upon incubation with rat plasma due to a higher concentration of esterase in plasma (See Figure 3.10). The hydrolysis of some esters (82a, 82b) was not measurable with rat plasma. Hydrolysis of derivatives in rat plasma showed a different dependence on the nature of the acid moiety of the carboxylic ester relative to pig liver esterase and rat brain homogenate. Generally, these ester analogs showed less hydrolysis by brain homogenate than by rat plasma and porcine liver esterase, possibly due to a lower esterase concentration in rat brain homogenate. Hydrolysis of the ester differed considerably among the prodrugs. The hydrolysis half-lives were

significantly dependent upon tissue and plasma concentration that controls the esterase concentration and the type of esterase.

The half-life ( $t_{1/2}$ ) for hydrolysis of the esters was determined in the presence of porcine liver esterase. The stability of the prodrugs toward hydrolysis by porcine liver esterase was significantly different. Some prodrugs of AZT (83f, 83g, 83i, 84d, 84e) were found to be relatively stable toward porcine liver esterase with half lives greater than 25 min at 37 °C. However, some prodrugs (84a, 84c, 84f, 85a, 91a-d, 92), which were found to be unstable, had a half-life less than 5 minutes.

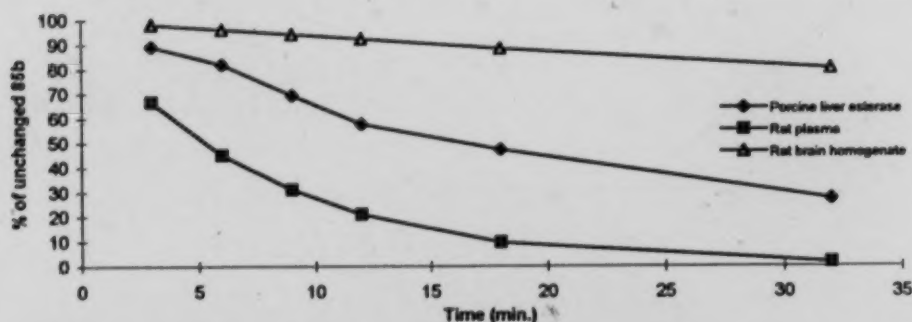


Figure 3.9. Stability of 85b upon *in vitro* incubation in several media at 37 °C.

The data in Tables 3.16.1 and 3.16.2 indicated that various esters of AZT were susceptible to the action of liver esterases with  $t_{1/2}$  in the range of 1.9 to 80.4 min. These results suggest that the esters 85b and 87 may have an increased plasma  $t_{1/2}$  *in vivo*.

### 3.3.2.3.2.2. Hydrolysis rate-anti HIV activity relationships

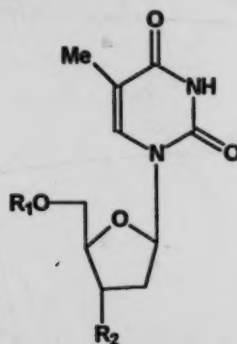
The structure-reactivity relationships for enzymatic hydrolysis of esters can be used to develop controlled release preparations of AZT *in vivo* by selecting an appropriate acyl group. There was a correlation between the capacity of the ester prodrug to undergo *in vitro* conversion to AZT and their anti-HIV potency. For example, compounds (82d, 83h) which undergo rapid conversion to AZT upon incubation with porcine liver esterase, rat plasma and brain homogenate exhibited anti-HIV activity comparable, or superior, to that

of AZT. In contrast, compounds (83g, 84d, 85b) which did not undergo conversion of AZT under these incubation conditions, exhibited a significantly decreased anti-HIV activity. On the other hand, some of the 3'-fluoro prodrug esters (91b-92) were found to undergo a greater % hydrolysis and showed more potent *in vitro* anti-HIV activity than that of corresponding 3'-azido derivatives (83e, 83f, 83h, 84b). The higher anti-HIV activity exhibited by these analogs could be due, at least in part, to fast release of FLT *in vitro*. For example 91d showed a higher potency ( $EC_{50} = 2.8$  nM) than 83h ( $EC_{50} = 36.6$  nM). Accordingly, 91d had a higher hydrolysis rate than 83h in porcine liver esterase and rat brain homogenate. The *in vitro* stability of these prodrug esters in RPMI/10% FBS (cell culture medium) must be known before any accurate conclusions can be made regarding the active anti-HIV species.

Pseudo-first-order half-lives, for ester hydrolysis by porcine liver esterase, were derived from the slope of semilogarithmic plots of ester concentration versus time (Tables 3.17.1 and 3.17.2, Figure 3.10). Although, some of these ester prodrugs showed more potent anti-HIV activity than others in this series, there was no correlation between potent activity and a slow ester hydrolysis rate (bioactivation) of prodrugs of 5'-O myristoyl derivatives of thymidine. Thus, the 2-bromomyristoyl ester of thymidine (81b), which has a  $t_{1/2} = 15.3$  min upon incubation with porcine liver esterase, was inactive against HIV infected CD4 lymphocytes. The longer half-life for compound 81b may be attributable to steric shielding by the  $\alpha$ -bromo atom of the ester moiety since bulky substituents such as bromo (Br) may shield the ester group from attack by the enzyme (Gallo *et al.*, 1988). The observed differences in the extent of ester hydrolysis (stability) are attributed to both steric and electronic variations in the fatty acid moiety of these ester compounds (81a-h).

The reactivity of the esterase depends remarkably on the acyl structure. The structure-reactivity relationships for enzymatic hydrolysis of the AZT and FLT prodrug esters can be used to develop controlled-release preparations of AZT or FLT *in vivo* by selecting an appropriate acyl group. It may be possible to control the rate and/or percentage of AZT and FLT release *in vivo*, which would influence the blood half-life and toxicity, by selecting the appropriate myristoyl analog moiety.

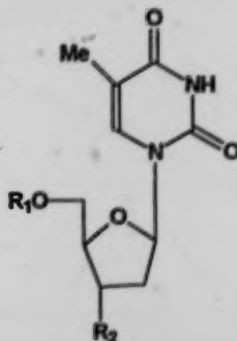
Table 3.16.1. Half-life for disappearance (*in vitro* hydrolysis) of 5'-O-myristoyl analogue derivatives of dThd (81a-h) and AZT (82a-83i) in the presence of porcine liver esterase, rat plasma, and rat brain homogenate.



| No. | R <sub>1</sub>                                                                      | R <sub>2</sub> | Porcine liver<br>esterase, <i>t</i> <sub>1/2</sub><br>(min) <sup>a</sup> | Rat plasma,<br><i>t</i> <sub>1/2</sub> (min) | Rat brain<br>homogenate,<br><i>t</i> <sub>1/2</sub> (min) |
|-----|-------------------------------------------------------------------------------------|----------------|--------------------------------------------------------------------------|----------------------------------------------|-----------------------------------------------------------|
| 81a | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                                 | OH             | 8.5                                                                      | ND                                           | ND                                                        |
| 81c | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO                           | OH             | 15.3                                                                     | ND                                           | ND                                                        |
| 81c | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                               | OH             | 8.5                                                                      | ND                                           | ND                                                        |
| 81d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | OH             | 11.9                                                                     | ND                                           | ND                                                        |
| 81e | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO                | OH             | ND <sup>b</sup>                                                          | ND                                           | ND                                                        |
| 81f | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(N <sub>3</sub> )CO              | OH             | 13.5                                                                     | ND                                           | ND                                                        |
| 81g | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                                  | OH             | 12.3                                                                     | ND                                           | ND                                                        |
| 81h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | OH             | 6.4                                                                      | ND                                           | ND                                                        |
| 82a | Cl(CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO               | N <sub>3</sub> | 13.0                                                                     | NM <sup>c</sup>                              | ND                                                        |
| 82b | N <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO  | N <sub>3</sub> | 17.4                                                                     | NM                                           | ND                                                        |
| 82c | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>9</sub> O(CH <sub>2</sub> ) <sub>2</sub> CO | N <sub>3</sub> | 17.9                                                                     | 3.77                                         | 19.2                                                      |
| 82d | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> O(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 9.3                                                                      | 1.6                                          | 48.9                                                      |
| 82e | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub> S(CH <sub>2</sub> ) <sub>7</sub> CO | N <sub>3</sub> | 6.8                                                                      | 1.7                                          | 44.5                                                      |
| 83a | F(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 6.2                                                                      | 2.3                                          | 35.0                                                      |
| 83b | I(CH <sub>2</sub> ) <sub>11</sub> CO                                                | N <sub>3</sub> | 14.7                                                                     | ND                                           | 35.0                                                      |
| 83c | Cl(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 7.1                                                                      | 1.1                                          | ND                                                        |
| 83d | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 11.2                                                                     | 1.4                                          | 46.5                                                      |
| 83e | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | N <sub>3</sub> | 5.2                                                                      | 1.1                                          | 20.6                                                      |
| 83f | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO                | N <sub>3</sub> | 25.6                                                                     | 3.6                                          | 103.0                                                     |
| 83g | Ph(CH <sub>2</sub> ) <sub>11</sub> CO                                               | N <sub>3</sub> | 33.7                                                                     | 2.7                                          | 69.4                                                      |
| 83h | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                                  | N <sub>3</sub> | 7.7                                                                      | 0.8                                          | 30.3                                                      |
| 83i | PhO(CH <sub>2</sub> ) <sub>11</sub> CO                                              | N <sub>3</sub> | 63.4                                                                     | ND                                           | 330.0                                                     |

(a) *t*<sub>1/2</sub> is the time required for 50% hydrolysis of the ester at 37 °C upon incubation with the specified esterase (mean, *n* = 3). In all cases the regression equation obtained had *r* = 0.99. (b) ND = Not determined. (c) NM = Not measurable (very susceptible, hydrolysis is so fast).

**Table 3.16.2.** Half-life for disappearance (*in vitro* hydrolysis) of 5'-*O*-myristoyl analogue derivatives of AZT (84a-87) and FLT (91a-93) in the presence of porcine liver esterase, rat plasma, and rat brain homogenate.



| No. | R <sub>1</sub>                                                       | R <sub>2</sub> | Porcine liver<br>esterase, <i>t</i> <sub>1/2</sub><br>(min) <sup>a</sup> | Rat plasma, <i>t</i> <sub>1/2</sub><br>(min) | Rat brain<br>homogenate,<br><i>t</i> <sub>1/2</sub> (min) |
|-----|----------------------------------------------------------------------|----------------|--------------------------------------------------------------------------|----------------------------------------------|-----------------------------------------------------------|
| 84a | MeO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> | 3.4                                                                      | 0.9                                          | 42.2                                                      |
| 84b | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | N <sub>3</sub> | 12.7                                                                     | ND <sup>b</sup>                              | 123.3                                                     |
| 84c | Br(CH <sub>2</sub> ) <sub>10</sub> CO                                | N <sub>3</sub> | 2.8                                                                      | 3.1                                          | 22.7                                                      |
| 84d | 4-I-PhO(CH <sub>2</sub> ) <sub>10</sub> CO                           | N <sub>3</sub> | 80.4                                                                     | 3.7                                          | 150.0                                                     |
| 84e | PhO(CH <sub>2</sub> ) <sub>10</sub> CO                               | N <sub>3</sub> | 50.7                                                                     | ND                                           | 53.1                                                      |
| 84f | N <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> CO                   | N <sub>3</sub> | 2.7                                                                      | 1.0                                          | 59.7                                                      |
| 85a | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(F)CO             | N <sub>3</sub> | NM <sup>c</sup>                                                          | 0.3                                          | 3.9                                                       |
| 85b | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CH(Br)CO            | N <sub>3</sub> | 16.8                                                                     | 5.3                                          | 100.6                                                     |
| 86  | Ph(CH <sub>2</sub> ) <sub>9</sub> CO                                 | N <sub>3</sub> | 10.2                                                                     | 2.9                                          | 28.0                                                      |
| 87  | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | N <sub>3</sub> | 18.1                                                                     | 10.0                                         | 34.8                                                      |
| 91a | Br(CH <sub>2</sub> ) <sub>11</sub> CO                                | F              | 1.9                                                                      | NM                                           | ND                                                        |
| 91b | MeO(CH <sub>2</sub> ) <sub>11</sub> CO                               | F              | 2.2                                                                      | 0.8                                          | 13.9                                                      |
| 91c | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>11</sub> CO | F              | 3.8                                                                      | 1.0                                          | ND                                                        |
| 91d | N <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> CO                   | F              | 4.4                                                                      | 0.8                                          | 21.9                                                      |
| 92  | CH <sub>3</sub> CH <sub>2</sub> S(CH <sub>2</sub> ) <sub>10</sub> CO | F              | 2.0                                                                      | 2.1                                          | 11.7                                                      |
| 93  | CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> CO                  | F              | 20.3                                                                     | 4.6                                          | 17.5                                                      |

(a) *t*<sub>1/2</sub> is the time required for 50% hydrolysis of the ester at 37 °C upon incubation with the specified esterase (mean, *n* = 3). In all cases the regression equation obtained had *r* = 0.99. (b) ND = Not determined. (c) NM = Not measurable (very susceptible, hydrolysis is so fast).



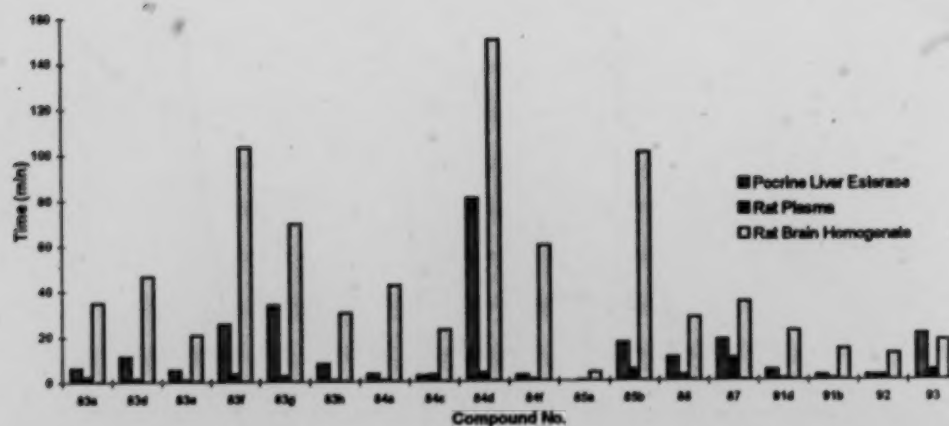


Figure 3.10. Comparative  $t_{1/2}$  values for some 5'-O-myristoyl analogue derivatives of dThd, AZT and FLT in the presence of porcine liver esterase, rat plasma, and rat brain homogenate.

#### 3.3.2.3.3. Chemical stability

Chemical stability on storage is a prerequisite for any drug. No significant measurable chemical hydrolysis of the 3'-azido-2',3'-dideoxy-5'-O-(myristoyl)thymidine (87) occurred during the time course of the experiment, as the substrate concentration did not change in control experiments where biological media had been replaced with an equal volume of buffer solution indicating that degradation of this prodrug was mediated predominantly by enzymatic processes. 3'-Azido-2',3'-dideoxy-5'-O-(myristoyl)thymidine had a  $t_{1/2}$  of 1575 min at 37 °C in phosphate buffer indicating that no chemical hydrolysis was observed using this control experiment in the absence of the enzyme. The same conclusion has been reported by Kawaguchi *et al.*, (1990). Various aliphatic 5'-esters of AZT (C2-AZT to C18-AZT) have been reported to be stable under moderate pH conditions (pH 4.0 and 7.0). No buffer catalysis was noted on hydrolysis at any pH value. All esters of 5'-O-AZT were unstable to strong acid and base hydrolysis (Kawaguchi 1985).

**3.3.2.3.4. The stability of 5'-O-(12-azidododecanoyl)thymidine (81h) and (±)-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) upon incubation with cell culture medium RPMI/10% FBS**

The culture medium was selected to understand the relationship between anti-HIV activity of some esters and their possible hydrolysis in culture medium during the anti-HIV testing assay. The hydrolysis reaction, which followed first order kinetics for **81h**, showed that most of the compound (60%) remained intact as the ester in the culture medium after incubation for six days. In the case of **85b**, no significant hydrolysis was observed even after six days of incubation. The less potent anti-HIV activity exhibited by **85b** compared to other prodrug esters evaluated could be due to slow intracellular and extracellular hydrolysis to AZT or a slow uptake into the CD4 infected cells in the culture medium.

**3.3.3. *In vivo* tissue distribution study of (±)-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) in Balb/c mice**

**3.3.3.1. Selection of prodrug**

In many cases it is difficult to design a prodrug with ideal properties such as a combination of adequate *in vitro* stability and facile conversion to the parent drug *in vivo*. Slow and/or rapid conversion of a prodrug into an active drug may provide inadequate delivery of a given drug to the target site. Therefore, using increased permeability as the sole basis to assess improvement in drug delivery via a prodrug may be an unacceptable or limited criterion. An alternative criterion is based upon the target organ containing a high level of a particular enzyme which is capable of selectively cleaving the prodrug moiety-drug linkage at that site (Stella, 1980). An important argument for the suitability of a compound to act as a prodrug is its mechanism of hydrolysis (enzyme type) and its kinetic properties under physiological conditions.

On the other hand, as mentioned before, AZT exhibits dose-related bone marrow toxicity, and to reduce this toxicity lower doses of AZT have been recommended (Volberding *et al.*, 1990). However, a lower dose may result in lower brain levels of AZT, thereby decreasing the therapeutic efficacy of AZT for treating cerebral infections. Prodrugs which have a longer residence time in the body and provide a sustained release

of the parent drug at concentrations adequate for its therapeutic anti-HIV efficacy, but not causing toxicity, are needed (Pizzo *et al.*, 1990). As discussed earlier, many ester prodrugs of AZT have been proposed, including 5'-fatty acid-AZTs. According to Kawaguchi *et al.* (1990), 5'-fatty acid-AZTs have been the most promising in producing a sustained release of AZT in plasma to date. AZT concentrations in plasma after ip administration of 5'-aliphatic acid-AZTs were shown to be more constant and persistent, especially when the acyl chain is increased to a C18 mostly as a result of longer retention due to its significantly increased lipophilicity and sustained enzymatic hydrolysis. Caproate and stearate AZT analogs in this series demonstrated a sustained release in plasma. The concentration of AZT decreased slowly and was retained up to 4 hours postdosing. The compounds (5'-aliphatic acid esters) showed a dramatic increase in lipophilicity and some compounds had Log P values greater than 5 in comparison with AZT (Log P = -0.31) in a chloroform:phosphate buffer system (Kawaguchi *et al.*, 1990).

Various prodrugs of AZT and FLT were therefore designed. The utility of 5'-esters of AZT and FLT as potential prodrugs is dependent upon their pharmacokinetic properties, tissue distribution, and rate of bioconversion to AZT and FLT. A sustained release of AZT from a prodrug is particularly important for drugs having narrow therapeutic indices. The intracellular bioavailability of the parent compound may differ, depending upon the rate of hydrolysis of the prodrug in T-lymphocytes or macrophages, the primary target cells for HIV replication. An ester analog of AZT with a moderate conversion rate to AZT may be designed by an appropriate combination of substituents present in the ester chain. Thus, design of an ester prodrug which is relatively stable to plasma esterases, and yet is hydrolyzed by the cellular enzymes of the target cells, appears to be a feasible approach for developing ester prodrugs.

Macrophages may concentrate large amounts of the lipophilic ester (Aggarwal *et al.*, 1990). This property would appear to make prodrug esters of AZT, and related compounds, promising candidate agents with an opportunity to target drug to the macrophage reservoir of the HIV infection, thereby reducing the toxicity of the antiviral nucleoside to other cells.

The *in vitro* anti-HIV assay results acquired suggested that compounds 3'-azido-2',3'-

dideoxy-5'-O-(4-oxatetradecanoyl)thymidine **82c** and 3'-azido-2',3'-dideoxy-5'-O-(12-bromododecanoyl)thymidine **83d** may have better clinical potential than AZT for the treatment of AIDS. However, they did not show high stability in the presence of rat plasma (Tables 3.16.1 and 3.16.2). The data obtained may help in the selection of an acyl function that releases the drug in a time and/or tissue-selective manner. Some of the prodrugs prepared are expected to possess good stability in plasma, and yet generate therapeutically effective levels of AZT in the target tissues. Although ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (**85b**) is less active *in vitro* than AZT, it may result in a larger percentage of the dose of the antiretroviral agent to be delivered to HIV-infected cells like macrophages. Compound (**85b**) appears to persist in the cells for long periods of time, relative to AZT, providing a depot of the antiviral prodrug that could release AZT slowly upon cellular metabolism. On the other hand, subsequent *in vivo* hydrolysis of **85b** to 2-bromomyristic acid (**68c**) may provide another benefit since **68c** was the most potent antifungal agent among the myristic acid analogs described earlier against *C. neoformans*, *C. albicans* and *A. niger* (see section 3.3.1.1.4). Thus, the 2-bromoester (**85b**) was selected for a pharmacokinetic study since it showed good *in vitro* stability in rat plasma and porcine liver esterase incubation studies. In addition, **85b** has a high partition coefficient suggesting its suitability for delivery of prodrug to macrophages in lymphoid tissues and the liver. The physiochemical properties of the target organ were both critical in predicting whether this prodrug (**85b**) is useful for the site-specific delivery of AZT to target sites (liver, brain). Since penetration through membranes is considered an important prodrug attribute, the prodrug was carefully chosen in order to ensure it possesses the required lipophilicity. However, the active drug should be selectively released at the targeted site (organ) to prevent undesirable or toxic effects. In addition to selectivity for the target tissue, the drug carrier should protect the pharmacological agent from the harsh physiological environment and reduce or abolish side-effects exhibited by the parent compound. In summary, this prodrug design concept provides an introduction to this area of drug research which suggests that more selective prodrugs can be designed by optimization of drug delivery using other myristoyl ester prodrugs.

**3.3.3.2. Pharmacokinetic studies of 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)**

An *in vivo* study using Balb/c mice was conducted by iv injection of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b), AZT (6) or 2-bromomyristic acid (68c) via the tail vein. The kinetics and biotransformation of these compounds (6, 68c, 85b) in male Balb/c mice were investigated as part of the thesis proposal (Tables 3.17 and 3.18, and Figures 3.14-3.18).

Mice received equimolar doses (0.31 mmol/kg) of the prodrug (85b, 169.8 mg/kg), AZT (6, 81.5 mg/kg) or 2-bromomyristic acid (68c, 93.7 mg/kg) via iv injection. Blood levels for AZT after administration of AZT (6) or 85b disappeared in an apparent biphasic manner which could be well fitted to a two compartment pharmacokinetic model (Figure 3.11) of the type:

$$C_t = Ae^{\alpha t} + Be^{\beta t}$$

where  $C_t$  is concentration at time  $t$ ,  $A$  is the distribution phase intercept,  $\alpha$  is the distribution phase slope,  $B$  is the elimination phase intercept,  $\beta$  is the elimination phase slope,  $e$  is the natural logarithm base, and  $t$  is time after injection. The adequacy of the model fit was judged, in all cases, by noting (1) the residual sums of squared deviations and (2) the degree of correlation between observed and predicted blood drug levels resulting from the model (Figure 3.12). The two-compartment model provides a description of the plasma pharmacokinetics for AZT and AZT released from the ester. Blood, liver and brain concentrations of AZT (6), the ester analog (85b) and 2-bromomyristic acid (68c) were determined by HPLC analysis. The pharmacokinetic parameters for regenerated AZT after iv doses of AZT (6) and ester (85b) were determined.



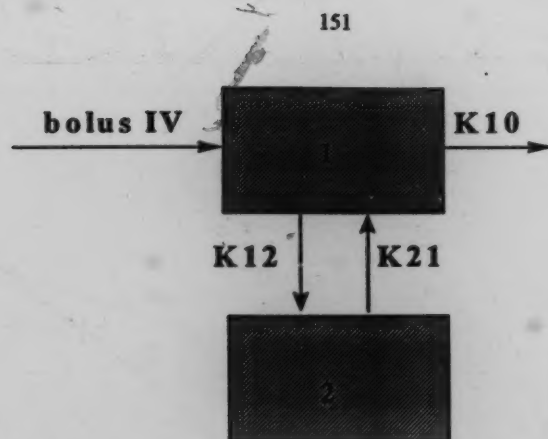


Figure 3.11. General diagram for two-compartment pharmacokinetic model with bolus input and first-order output.

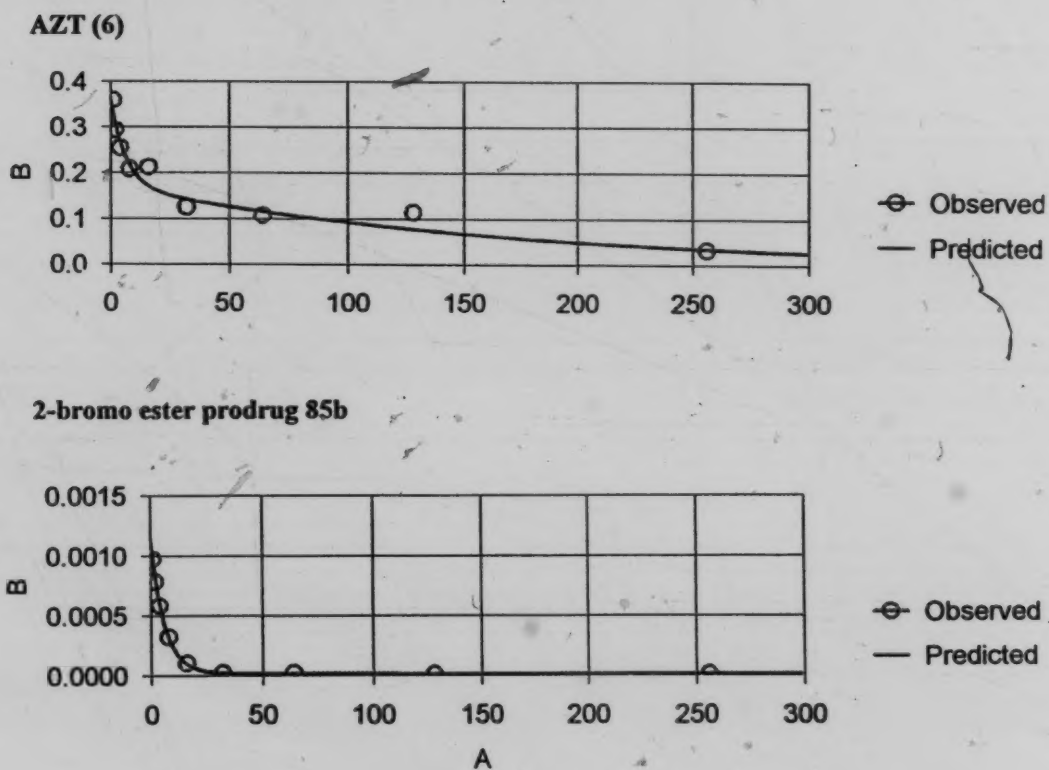


Figure 3.12. Plot of observed and predicted values for the mean concentrations of 85b and 6 in blood (Y axis) after a 0.31 mmol/kg iv bolus injection into mice vs time (X axis).

### 3.3.3.2.1. HPLC chromatograms

Figure 3.13 illustrates representative chromatograms obtained under the conditions of analysis. It was necessary to develop a gradient elution HPLC method to separate the ester (85b), its metabolite, AZT, and internal standard. The HPLC conditions described in experimental sections 4.11.1, 4.11.2 provided adequate resolution of the above compounds.

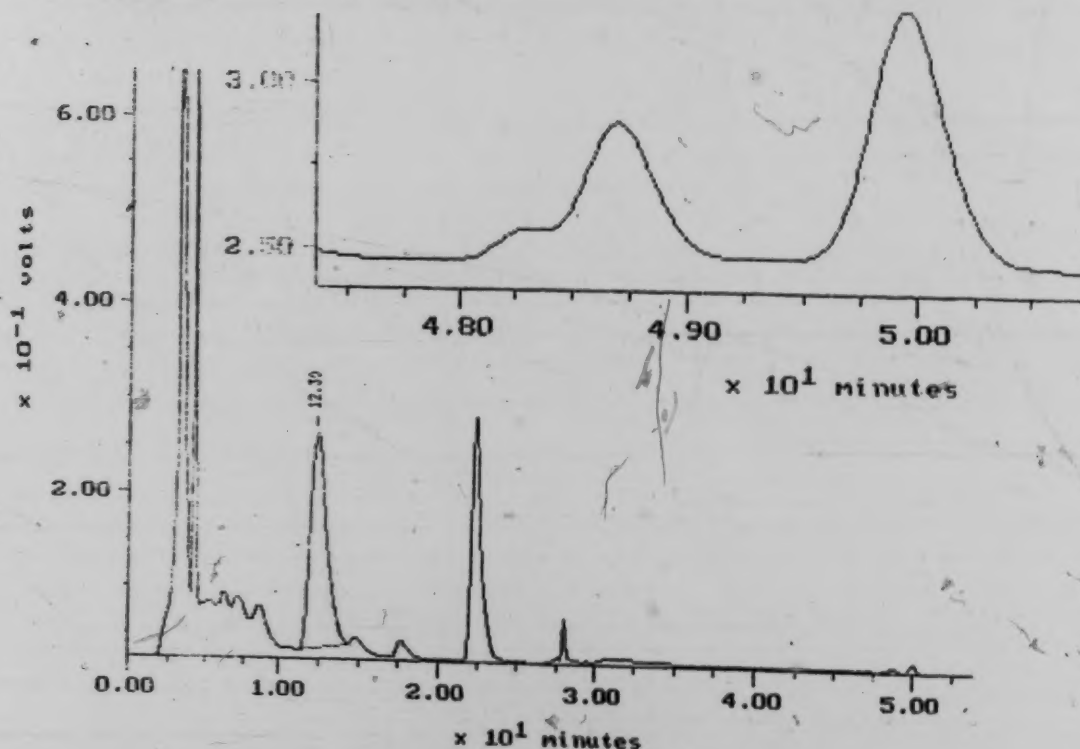


**Figure 3.13.** HPLC analysis of mouse liver extracts after injection of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b). Retention time (min): 5-bromo-2'-deoxyuridine (internal standard) (a, 12.3), AZT (6) (b, 22.3), and 2-bromoester (85b) (c, 49.9). Sample obtained at 1 min from the liver after iv administration of 169.8 mg/kg of the ester.

An evaporative light scattering detector was used for the detection of 2-bromomyristic

acid (68c) in the blood, brain and liver using an HPLC gradient system mentioned in experimental section 4.7.2.1.4.

The area under the curve for blood, brain and liver concentration vs time (AUC) was calculated using the WinNonlin program, with extrapolation to infinity using the least squares terminal disposition rate constant ( $\lambda_z$ ). The terminal disposition rate constant was equal to the slope of the equation obtained from linear regression of the natural Log of the concentration and time values in the terminal phase. The elimination half-life ( $t_{1/2}$ ) was calculated from  $0.693\lambda_z^{-1}$ . The brain:blood AZT concentration ratio was calculated from  $AUC_{\text{brain}}/AUC_{\text{blood}}$ .

### 3.3.3.2.2. Pharmacokinetic parameters

Whole blood was used for calculation of pharmacokinetic parameters in this study. The log blood concentration for AZT and the ester after an iv dose decreased biexponentially with time. Two compartmental pharmacokinetic parameters were estimated by the WinNonlin program using a two compartmental iv bolus model. Pharmacokinetic parameters, presented in Tables 3.17 and 3.18, showed an  $\alpha$ -phase half-life (4.2 min) and a  $\beta$ -phase elimination half-life (428.5 min) for injected ( $\pm$ )-3'-Azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) compared to a  $\alpha$   $t_{1/2}$  4.4 min and a  $\beta$   $t_{1/2}$  of 112.5 min (1.87 h) for injected AZT. The short distribution half-life for the prodrug shows that early blood concentrations are a crucial determinant of pharmacokinetic constants for the prodrug. The rapid *in vivo* clearance of the prodrug from blood corresponds to its *in vivo* distribution within the body and extraction by other tissues such as liver and fat. AZT administration generated high initial blood levels of the AZT which decreased rapidly as a function of time.

The mean residence time (MRT) for the prodrug was larger than that for injected AZT. The prodrug had a MRT of 379.03 min compared to AZT with a MRT of 155.92 min.

**Table 3.17.** Parameters obtained from two-compartment modeling of the blood clearance curves of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomystoyl)thymidine (85b) and 3'-azido-2',3'-dideoxythymidine (6) in Balb/c mice.

| Parameter <sup>a</sup>                                       | Prodrug<br>85b   | ester<br>AZT     |
|--------------------------------------------------------------|------------------|------------------|
| AUC ( $\mu\text{mol.g}^{-1}.\text{min}$ ) <sup>b</sup>       | 17.34 $\pm$ 4.69 | 29.12 $\pm$ 2.92 |
| AUC (AZT) ( $\mu\text{mol.g}^{-1}.\text{min}$ ) <sup>c</sup> | 12.28 $\pm$ 0.76 | 29.12 $\pm$ 2.92 |
| K10-half-life (min) <sup>d</sup>                             | 10.67            | 56.38            |
| Alpha phase half-life (min) <sup>e</sup>                     | 4.23             | 4.42             |
| Beta phase half-life (min) <sup>f</sup>                      | 428.50           | 112.48           |
| K10 ( $\text{min}^{-1}$ ) <sup>g</sup>                       | 0.065            | 0.012            |
| K12 ( $\text{min}^{-1}$ ) <sup>h</sup>                       | 0.096            | 0.070            |
| K21 ( $\text{min}^{-1}$ ) <sup>i</sup>                       | 0.004            | 0.078            |
| C <sub>max</sub> ( $\mu\text{mol/g}$ ) <sup>j</sup>          | 1.12 $\pm$ 0.09  | 0.36 $\pm$ 0.07  |
| AUMC ( $\mu\text{mol.g}^{-1}.\text{min}^2$ ) <sup>k</sup>    | 6574.02          | 4540.96          |
| MRT (min) <sup>l</sup>                                       | 379.03           | 155.92           |
| V <sub>ss</sub> (mL) <sup>m</sup>                            | 183.27           | 44.90            |
| CL ( $\text{mL min}^{-1}.\text{kg}^{-1}$ ) <sup>n</sup>      | 17.58 $\pm$ 4.76 | 10.47 $\pm$ 1.05 |

<sup>a</sup> Calculated using the WinNonlin program. <sup>b</sup> Area under the curve of blood concentration vs time extrapolated to infinity. <sup>c</sup> Area under the blood concentration curve vs time extrapolated to infinity for AZT produced from 85b or 6. <sup>d</sup> The half-life associated with the rate constant K10. <sup>e</sup> Half-life of the distribution phase. <sup>f</sup> Half-life of the elimination phase. <sup>g</sup> The rate at which the drug leaves the system from the central compartment (the elimination rate). <sup>h</sup> Transfer rate, 1 to 2. <sup>i</sup> Transfer rate, 2 to 1. <sup>j</sup> The peak or maximum concentration. <sup>k</sup> Area under the moment curve. <sup>l</sup> Mean residence time is the average amount of time a drug remains in a compartment or system. <sup>m</sup> Volume of distribution at steady state ( $V_{ss} = \text{MRT} \cdot \text{CL}$ ). <sup>n</sup> Blood clearance.

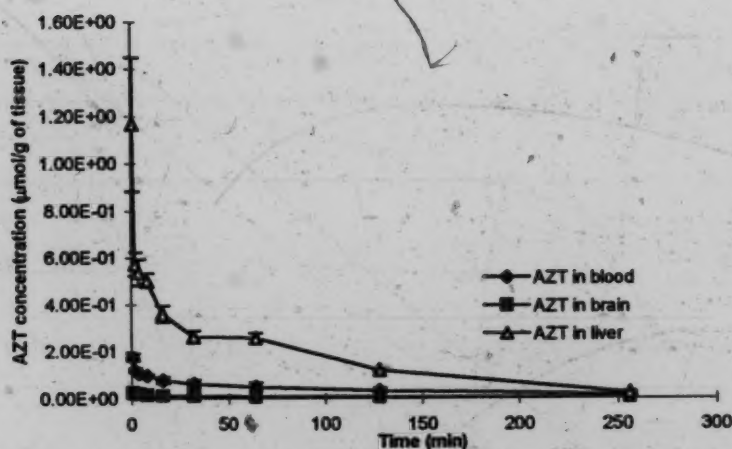


Figure 3.14. Concentrations ( $\mu\text{mol/g}$ ) of AZT (6) in blood, brain and liver after iv tail vein injection of 169.8 mg/kg of  $(\pm)$ -3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

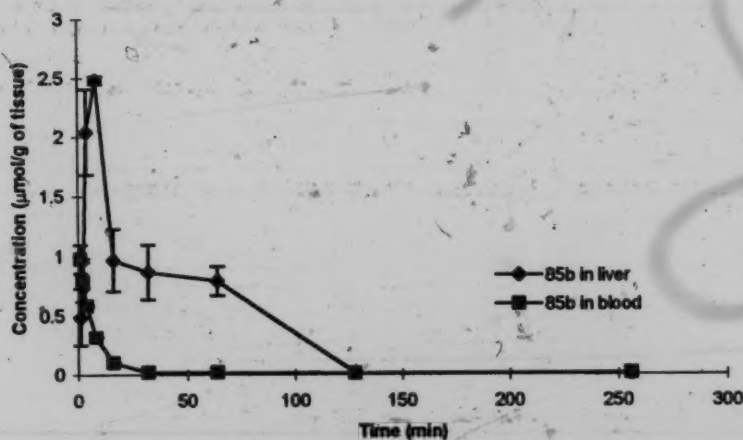


Figure 3.15. Concentrations ( $\mu\text{mol/g}$ ) of  $(\pm)$ -3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) in blood and liver after iv tail vein injection of 169.8 mg/kg of  $(\pm)$ -3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).



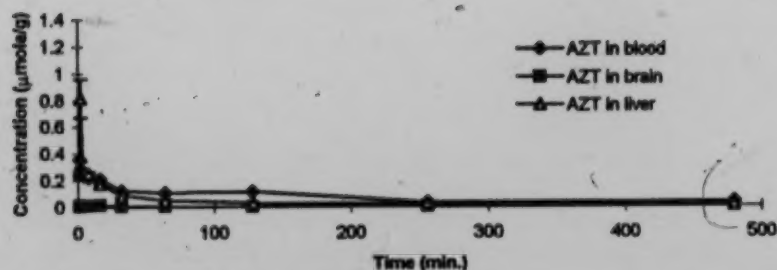


Figure 3.16. Concentrations ( $\mu\text{mol/g}$ ) of AZT in the liver, brain and blood after iv tail vein injection of 81.5 mg/kg of 3'-azido-2',3'-dideoxythymidine (6) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

The long residence time for the ester in liver and blood might suggest a sustained release of AZT from the prodrug. Sustained release of AZT from the ester should provide an added advantage over AZT alone in blood and liver by reducing the frequency of drug administration and increasing the peripheral tissue drug distribution.

### 3.3.3.3. Tissue (blood, liver brain) kinetics of 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

In this section the biodistribution and kinetic parameters for 6, 68c and 85b in the blood, liver and brain are discussed (Table 3.18, Figure 3.17).

**Table 3.18.** Important pharmacokinetic parameters for ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) and 3'-azido-2',3'-dideoxythymidine (6) (From 85b or 6) in mouse blood, brain and liver.

| Tissue | AZT<br>or Ester   | C <sub>max</sub><br>( $\mu$ mol/g) <sup>a</sup> | t <sub>max</sub><br>(min) <sup>b</sup> | AUC <sup>c</sup><br>( $\mu$ mol.min/g) |
|--------|-------------------|-------------------------------------------------|----------------------------------------|----------------------------------------|
| Blood  | AZT               | 0.36 $\pm$ 0.07                                 | 1                                      | 29.12 $\pm$ 2.92                       |
|        | Ester             | 1.12 $\pm$ 0.09                                 | 1                                      | 17.34 $\pm$ 4.69                       |
|        | AZT<br>(produced) | 0.17 $\pm$ 0.02                                 | 1                                      | 12.28 $\pm$ 0.76                       |
| Brain  | AZT               | 0.01 $\pm$ 0.0008                               | 1                                      | 1.36 $\pm$ 0.23                        |
|        | Ester             | ND <sup>d</sup>                                 | ND <sup>d</sup>                        | ND <sup>d</sup>                        |
|        | AZT<br>(produced) | 0.03 $\pm$ 0.002                                | 1                                      | 2.81 $\pm$ 0.50                        |
| Liver  | AZT               | 0.81 $\pm$ 0.14                                 | 2                                      | 12.70 $\pm$ 2.71                       |
|        | Ester             | 2.48 $\pm$ 0.03                                 | 8                                      | 26.16 $\pm$ 8.13                       |
|        | AZT<br>(produced) | 1.16 $\pm$ 0.28                                 | 1                                      | 42.48 $\pm$ 4.06                       |

<sup>a</sup> The peak or maximum concentration. <sup>b</sup> The time of peak concentration. <sup>c</sup> Area under the concentration vs time curves (0  $\rightarrow$  infinity). <sup>d</sup> ND = not detectable.

#### 3.3.3.3.1. Blood kinetics for 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

The blood levels of AZT (6) and ester (85b) after iv injection of the ester are presented in Tables 3.19 and 3.20, and Figures 3.14-3.16.

##### 3.3.3.3.1.1. ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

Kawaguchi *et al.*, (1990, 1991) reported that the plasma concentration of caproate-AZT and stearate-AZT following intraperitoneal administration of the caproate or stearate

esters to mice was below the detection limit. The absence of caproate and stearate in the plasma may be attributable to the high hydrophobicity or favorable tissue distribution of these esters. Several 5'-O-esters of AZT such as C2-AZT, C10-AZT and C-18-AZT could not also be detected in rat blood after iv administration of prodrugs (Kawaguchi *et al.*, 1991, Hasegawa *et al.*, 1993, Kawaguchi *et al.*, 1990, 1992). The absorption into certain tissues, such as erythrocytes or fatty tissues may be responsible for the absence of these esters in plasma since fatty tissues can act as reservoirs for the prodrugs.

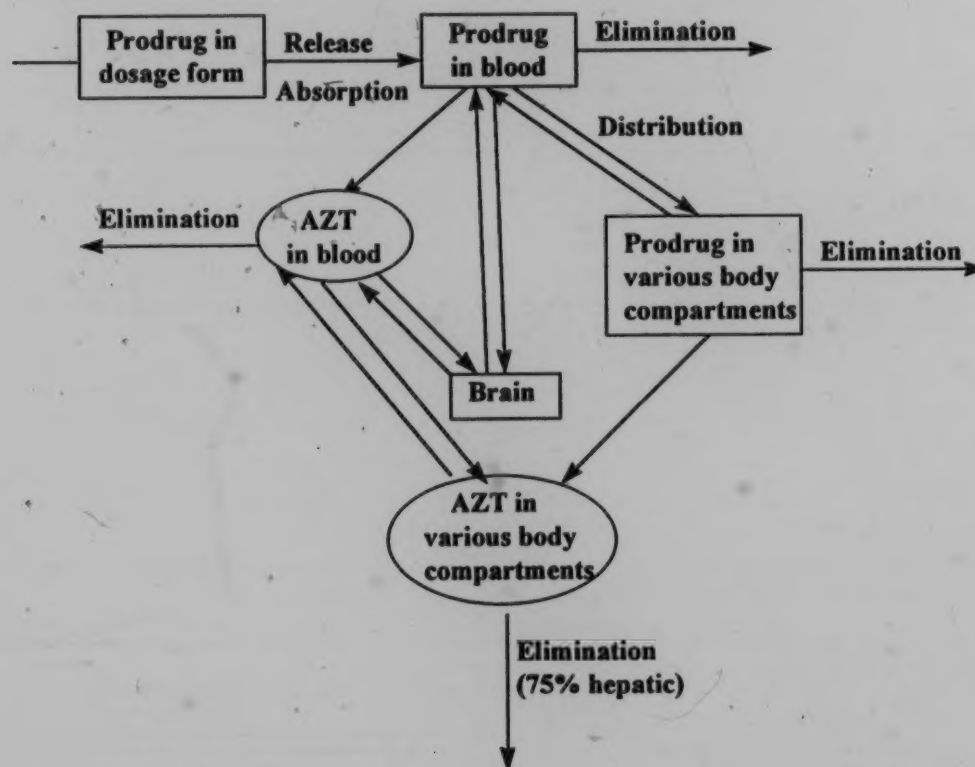


Figure 3.17. Diagram of the distribution of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine after iv administration of 169.8 mg/kg of the prodrug.

Table 3.19. Concentrations ( $\mu\text{mol/g}$ ) of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomethylthymine)thymine (85b) in liver and blood after tail vein injection of 169.8 mg/kg of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomethylthymine)thymine (85b) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

| Tissue | 1 min           | 2 min           | 4 min            | 8 min            | 16 min           | 32 min           | 64 min           | 128 min          | 256 min            |
|--------|-----------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|--------------------|
| Liver  | 0.49 $\pm$ 0.24 | 0.74 $\pm$ 0.24 | 2.04 $\pm$ 0.36  | 2.48 $\pm$ 0.03  | 0.97 $\pm$ 0.26  | 0.87 $\pm$ 0.23  | 0.79 $\pm$ 0.12  | 0.01 $\pm$ 0.005 | 0.006 $\pm$ 0.0001 |
| Blood  | 0.98 $\pm$ 0.12 | 0.79 $\pm$ 0.17 | 0.56 $\pm$ 0.008 | 0.32 $\pm$ 0.002 | 0.10 $\pm$ 0.002 | 0.02 $\pm$ 0.003 | 0.02 $\pm$ 0.004 | 0.01 $\pm$ 0.002 | 0.019 $\pm$ 0.003  |

Table 3.20. Concentrations ( $\mu\text{mol/g}$ ) of AZT in liver, brain and blood after iv tail vein injection of 169.8 mg/kg of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomethylthymine)thymine (85b) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

| Tissue            | 1 min             | 2 min             | 4 min             | 8 min             | 16 min            | 32 min            | 64 min             | 128 min            | 256 min            |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------|--------------------|--------------------|
| Liver             | 1.16 $\pm$ 0.28   | 0.60 $\pm$ 0.05   | 0.57 $\pm$ 0.05   | 0.53 $\pm$ 0.03   | 0.36 $\pm$ 0.03   | 0.26 $\pm$ 0.03   | 0.26 $\pm$ 0.02    | 0.12 $\pm$ 0.003   | 0.024 $\pm$ 0.0007 |
| Brain             | 0.025 $\pm$ 0.002 | 0.021 $\pm$ 0.005 | 0.019 $\pm$ 0.004 | 0.015 $\pm$ 0.001 | 0.012 $\pm$ 0.002 | 0.01 $\pm$ 0.0017 | 0.006 $\pm$ 0.0008 | 0.005 $\pm$ 0.0003 | 0.004 $\pm$ 0.001  |
| Blood             | 0.17 $\pm$ 0.02   | 0.12 $\pm$ 0.01   | 0.11 $\pm$ 0.005  | 0.098 $\pm$ 0.006 | 0.08 $\pm$ 0.01   | 0.06 $\pm$ 0.02   | 0.05 $\pm$ 0.02    | 0.03 $\pm$ 0.01    | 0.02 $\pm$ 0.00    |
| Brain/blood ratio | 0.144             | 0.176             | 0.179             | 0.154             | 0.156             | 0.165             | 0.125              | 0.163              | 0.258              |

Drug having a larger  $P$  value have a greater distribution of the drug into a lipid phase (i.e., membranes). In general, as  $P$  approaches infinity, the drug interaction will become so great, so it will localize in the first lipophilic phase with which it comes into contact. A compound with an excessive lipid solubility and no protein binding in blood will no longer circulate in the bloodstream but this was not the case for the 5'- $O$ -(2-bromomyristoyl) analog of AZT (85b). It was possible to detect the prodrug ester (85b) in mouse blood. Therefore other factors such as stability of the 5'- $O$ - ester in plasma has a major role in their retention in plasma. High lipophilicity may result in distribution of the lipophilic esters to peripheral fatty tissues. However, an equilibrium will be obtained for the drug between the blood and these tissues in the case of stable esters such as 85b. A significant fraction of the prodrug was rapidly hydrolyzed, as demonstrated by the presence of 0.17  $\mu\text{mol/g}$  of AZT in blood at 1 min. Most of the ester will be distributed to tissues even at 1 min postinjection or hydrolyzed to AZT. Only 11.68% of the ester was detected at 1 min in blood. However, the ester could still be detected in blood at 480 min, indicating there is an equilibrium for 85b between peripheral tissues and blood. Rapid changes in the level of AZT in plasma may be due to rapid distribution of the prodrug from blood before hydrolysis to AZT occurs. The redistribution of free AZT (6) and prodrug ester (85b) back into blood from peripheral tissues that accumulated the ester may be responsible for the characteristic slow decrease in the level of AZT after 16 min.

The half-life of the terminal phase for 85b is considerably longer than the reported value for AZT. This observation indicates that the  $t_{1/2}$  for the ester was longer than might be expected based on the *in vitro* incubation experiments.

After injection of 85b into mice, there is a finite time required for dispersion. After enzymatic hydrolysis to the free drug, it will maintain relatively low levels of the active drug for long periods of time. This plasma-time profile suggested the long retention and sustained enzymatic hydrolysis of the ester in the body that eventually resulted in a long residence time for AZT in plasma. The longer residence time of AZT following iv administration of ester (85b) may be attributable at least in part to its relatively low specificity to enzymatic hydrolysis.



### 3.3.3.3.1.2. 2-Bromomyristic acid (68c)

The fatty acid 2-bromomyristic acid (68c) was not detected following administration of intact fatty acid (68c) via tail vein injection since the fatty acid has a high rate of blood clearance. Absorption into certain tissues such as erythrocytes, fatty tissues or binding to blood proteins or fast metabolism may be responsible for the absence of fatty acid in blood. 68c was detected up to 2 min post-dosing at a  $0.1 \mu\text{mol/g}$  concentration following the administration of 2-bromomyristoyl ester (85b) via tail vein injection.

### 3.3.3.3.1.3. AZT (6)

Kawaguchi *et al.* (1990) used three of the prodrugs to AZT (acetate, caproate, and stearate) that were administered to mice intraperitoneally and plasma concentrations of AZT were measured. The AZT concentration in plasma following the acetate prodrug administration rapidly decreased with a half-life ( $t_{1/2}$ ) of 14.5 min, which is similar to that demonstrated for direct AZT administration ( $t_{1/2} = 11.7$  min). However, the AZT concentrations following the caproate or stearate administration decreased slowly and were maintained for as long as 4 h after dosing (2 h for AZT or AZT acetate) (Kawaguchi *et al.*, 1990). Their results suggest that AZT concentrations following administration of aliphatic ester prodrugs are dependent upon the length of the acyl chain, which alters the rate of hydrolysis and elimination from the body, and that an optimal sustained release of AZT may be obtained by selecting an appropriate acyl group (Kawaguchi *et al.*, 1990).

AZT was detected and quantified as a primary metabolite of the prodrug. ( $\pm$ )-3'-Azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) converted rapidly to AZT after iv injection and afforded a high concentration of AZT in blood samples. The AUC for AZT, as a metabolite of the prodrug ester (85b) was ( $12.28 \pm 2.93 \mu\text{mol}\cdot\text{min/g}$ ) less than observed when an equimolar dose of AZT itself was injected ( $29.12 \pm 2.92 \mu\text{mol}\cdot\text{min/g}$ ). The molar concentration for 85b was lower than the molar concentration for AZT in blood from AZT injected at longer time points that reconfirms the lower molar concentration for AZT produced from 85b, compared to AZT from AZT administration (Figure 3.18). Constant lower concentrations of AZT (6) were observed in plasma after prodrug, compared to those after AZT (6), dosing that may lower the blood side effects of

AZT similar to a dihydropyridine chemical delivery system (see section 1.5.2.2.1.3). For example, the level of AZT in blood was 0.36 and 0.17  $\mu\text{mol/g}$  at a 1 min post-injection time for the same molar dose of either AZT (6) and its ester (85b), respectively.

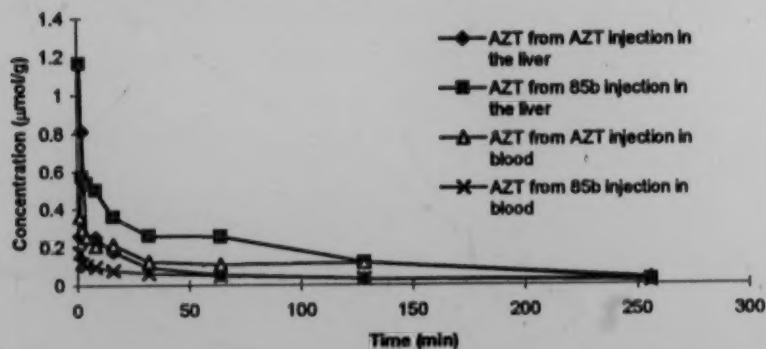


Figure 3.18. Comparative concentrations of AZT in blood and liver after iv tail vein injection for a 169.8 mg/kg dose of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) and a 81.5 mg/kg dose of 3'-azido-2',3'-dideoxydeoxythymidine (6) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

### 3.3.3.3.2. Liver kinetics of 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

To treat hepatitis B more effectively, it would be desirable to direct a larger fraction of administered antiviral nucleoside to the liver because some of these agents are toxic to nonhepatic tissues and may not achieve adequate levels in liver tissue. Targeting the ester prodrug of anti-HBV nucleosides to the liver is a general approach which can be applied to any antiviral nucleoside. Many nucleosides active against HBV are known to be very toxic *in vivo*, and the ester prodrug targeted to the liver may be expected to reduce drug toxicity and improve efficacy if the liver itself is not the toxicity target. The ester moiety may also protect the 5'-OH of AZT from glucuronidation, thus increasing the half-life of AZT. Fifteen to twenty percent of zidovudine (AZT) is excreted unchanged into the urine, and seventy five percent is metabolized by hepatic glucuronidation to form 3'-azido-2',3'-dideoxy-5'-O-glucuronyl thymidine (an inert metabolite). Since derivatives such as 5'-O-esters of AZT have an ester linkage at this 5' position, the same pathway will not

metabolize them. This will increase the effective liver concentration and allow a lower dose of active drug to achieve the same therapeutic benefit.

#### 3.3.3.3.2.1. ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

The 2-bromoester (85b) is present in the liver at a higher concentration than in the blood. This accumulation may occur by binding to the tissue more extensively than to plasma proteins. The molar concentration of ester in liver was much higher than in blood between 4-128 min (Figure 3.18). At 8 min, 29.6% of the administered ester was recovered from liver. This indicates that parenteral administration of the ester prodrug to AZT may increase the *in vivo* efficacy and selectivity of nucleoside therapy for hepatitis B infection by delivering a much larger fraction of the administered drug to the liver. Further studies are required to evaluate what proportion of the dose is delivered to hepatic parenchymal cells vs endothelial and Kupfer cells *in vivo*.

Other anti-HBV agents produced from these ester prodrugs *in vivo* may be taken up by the liver cells which have been found to harbor the target virus. Based on the information gained from the inhibition of DNA production of HBV-infected cells in tissue culture (see section 3.3.2.1.2), and the targeted delivery in mice, the fatty acid derivatives described in this thesis project may offer a potentially effective therapeutic agent for the treatment of hepatitis B infections. Although further studies are needed to clarify the distribution and kinetics of the AZT esters investigated, the results acquired in this study suggest that adequate delivery and sustained levels of AZT in liver can be achieved by selecting an appropriate ester derivative of AZT.

Similar results were obtained by Hostetler when lipid derivatives of dideoxycytidine (5) and azidothymidine (6) like 1,2-dimyristoylglycerophospho-5'-(2',3'-dideoxy)cytidine were taken up in the liver in very large amounts (Hostetler, 1993a). Higher concentrations were observed in plasma, liver, lymph nodes and the spleen using the phosphatidyl ddC rather than ddC (5). The AUC in liver for phosphatidyl-ddC was 42 times greater than that for ddC (5).

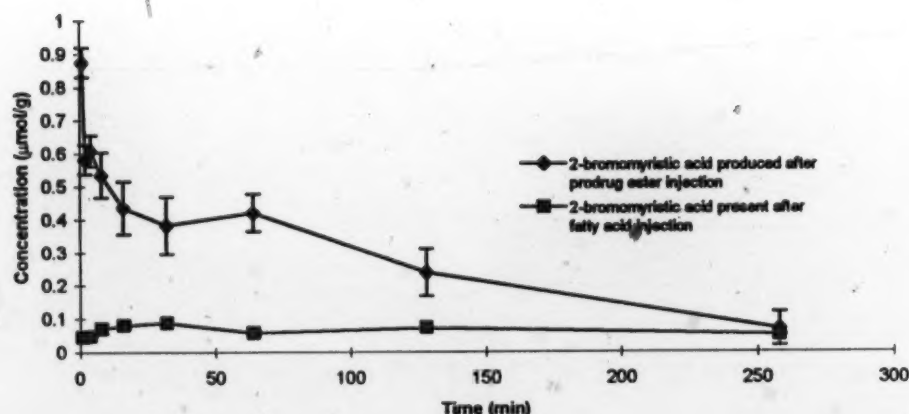


**Figure 3.19.** Concentrations of 2-bromomyristic acid in liver after iv injection of 93.7 mg/kg of 2-bromomyristic acid (**68c**) or 169.8 mg/kg of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (**85b**) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

#### 3.3.3.3.2.2. 2-Bromomyristic acid (**68c**)

The concentration of **68c** in the liver, following administration of the ester (**85b**), was greater than that from administered 2-bromomyristic acid (**68c**) due to a higher extraction of **85b**, relative to **68c**, in the liver. It appears that most of **68c** was metabolized by different pathways before reaching the liver or metabolized once in the liver (Figure 3.19). The absence of 2-bromomyristic acid in blood after injection of **68c** into mice confirmed this postulation.

The metabolism of 2-bromotetradecanoic acid (**68c**) in the liver has not been investigated. A peak was detected with a HPLC retention time of approximately 27 min, which is similar to that for 2-bromotetradecanoic acid, is assumed to be 2-bromotetradecanoic acid.

Although the effect of bromine at the 2-position of fatty acid esters has not yet been reported, 2-bromo substituted fatty acids are believed to undergo slower  $\beta$ -oxidation. Thus, the 2-bromo fatty acids possess at least one potential advantage over the non-

brominated analogs, a quality which would be useful for systematic antifungal activity.

### 3.3.3.3.2.3. AZT (6)

The concentration of AZT in the liver after injection of the prodrug (85b) was higher than after injection of AZT, which indicated that this prodrug underwent higher hepatic extraction than AZT (Figure 3.18). It was shown that this lipophilic prodrug to AZT (85b) accumulates to a 4.5 fold-higher level in the liver than free AZT after 1 min and 4.8 fold higher after 64 min.

The extensive distribution of the test compound into liver, particularly at very early time periods, is likely due in part to its high lipophilicity. The AUC for AZT produced after 85b dosing in liver ( $AUC = 42.48 \pm 4.06 \mu\text{mol}\cdot\text{min/mL}$ ) is larger than that when AZT was injected ( $17.70 \pm 2.71 \mu\text{mol}\cdot\text{min/mL}$ ).

### 3.3.3.3.3. Brain uptake of 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

The ability of AZT to cross the blood-brain-barrier (BBB) is less than optimal. Therefore, AZT does not effectively suppress viral replication in the brain. Theoretically, a higher central nervous system concentration could be achieved by raising the plasma levels of AZT. However, due to AZT's dose-related bone marrow toxicity, increasing brain uptake by increasing the administered dose is not recommended. Moreover, it has been shown that bulk efflux of AZT from CSF and the brain via the organic anion transport system plays an important role in the observed limited AZT levels in CSF and the brain.

It was of interest to investigate the properties of an agent which can accumulate in the brain, thereby providing a sustained release of the antiviral agent to maintain a therapeutically effective concentration. Many investigations have focused on the development of agents which can more readily penetrate the CNS by readily crossing the blood-brain barrier. These studies involved modification of the 5'-hydroxyl group of parent anti-HIV nucleosides with different groups such as phosphate or fatty acyl groups (Bodor *et al.*, 1981a, b, Aggarwal *et al.*, 1990, Kawaguchi *et al.*, 1990, Palomino *et al.*, 1989, Chu *et al.*, 1990, Henin, *et al.*, 1991), modification of the nucleoside base with



lipophilic functional groups (Palomino *et al.*, 1990, Barchi *et al.*, 1991) or using a chemical delivery system (Bodor *et al.*, 1992, Torrence *et al.*, 1993). AZT-DP with a partition coefficient of 4.8 resulted in a 50% increase in cellular uptake compared with AZT (Gogu *et al.*, 1989). Also, most 5'-ester prodrugs with higher partition coefficients (7.2-17.9) reported by Aggarwal *et al.*, demonstrated increased cellular uptake (up to four-fold higher than AZT). Results from *in vitro* and *in vivo* evaluation of ester prodrugs have shown improved site delivery and/or pharmacokinetic parameters for the parent nucleoside. However, these modifications have so far not resulted in compounds with a clear-cut therapeutic efficacy.

A systematic effort has been made in our laboratory to synthesize a series of prodrugs to AZT and FLT to overcome their limited penetration across the BBB. In an attempt to develop derivatives of AZT which might be sequestered in the central nervous system and release AZT, ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) was selected for study. Cleavage of the ester group may then release AZT (6) and 2-bromomyristic acid (68c). On the other hand, the enhanced lipophilicity of this analog (85b) may enable it to enter cells more readily by diffusion. The high blood stability, in conjunction with its enhanced lipophilicity, suggests that this compound should provide a higher concentration in the brain than AZT. This postulate is based on the observation that brain capillary permeability is often related to the *n*-octanol-water partition coefficient of compounds (Levin 1980).

The prodrug-to-drug conversion may take place before brain absorption, during absorption, after absorption or at a specific site in brain, are all dependent upon the specific goal for which the prodrug is designed. If the objective of the prodrug is to produce a sustained drug action through rate-limiting prodrug conversion, the rate of the conversion should not be too high which is the case for the 2-bromomyristoyl ester (85b).

No prodrug ester 85b was detected in brain samples and the level of AZT in the brain was lower than in the blood and liver. Since no significant quantity of the ester could be detected in the brain sample, no graph depicting ester brain concentration was acquired in this study. In order to offer an explanation for the low concentration of the ester in the brain, several hypotheses can be put forward for consideration as follows. The results from

*in vivo* administration of 2-bromomyristic acid, AZT and prodrug ester will help to clarify which one of these hypotheses are correct.

(1) First of all, the inability to detect **85b** in brain can be attributed to the fact that the lipophilic ester might have egressed from the brain before hydrolysis. High lipophilicity, which facilitates its entry, likewise facilitates its exit. The major drawback is that while influx to the CNS is increased, the efflux is also greater such that; the ester has poor retention in the CNS. *In vitro* incubation studies of prodrug **85b** in rat plasma and rat brain homogenate indicated that rates of blood conversion from prodrug to parent drug in brain homogenate were less than in plasma.

(2) Alternatively, the ester **85b** might have entered the brain, but was partially hydrolyzed to liberate AZT before complete efflux. The brain concentration of prodrug **85b** was below the limit of quantification. Tsuzuki *et al.*, (1994) showed that AZT-C18 can be detected in rat brain after iv administration at 2 h postinjection in spite of its high lipophilicity. The presence of the ester **85b** after administration in the brain was not detectable even after 1 min due to the limited sensitivity (approximately 0.5 µg/mL) of HPLC-UV detection. It was assumed that the ester was rapidly delivered to the brain where it could be partially hydrolyzed to AZT. Although the prodrug **85b** may diffuse out of cells at the same rate at which it diffuses into cells, enzymatic hydrolysis of the 5'-O-ester moiety will afford the more polar and less lipophilic AZT that should be retained in the cell to a greater extent. The subsequent conversion of AZT to its highly hydrophilic 5'-O-monophosphate derivative by thymidine kinase would result in complete trapping.

(3) It was postulated that possible diffusion of these highly lipophilic prodrugs following bolus injection into the relatively small tail vein of mice may have reduced the amount of the injected test compound in the circulation, at least for the early post injection time periods. This may finally have resulted in a reduction of the ester concentration in brain samples.

(4) Another possibility is that the prodrug **85b** did not cross BBB. The absence of prodrug in the CNS may reflect poor uptake of prodrug ester. In the absence of an active transport system, the enhanced lipophilicity of the prodrug should facilitate the entry into cells and the ability to cross the BBB more readily by diffusion than AZT. However, it was

reported that for drugs which have high *n*-octanol/water *P* values, their rate of BBB passage will be limited by the blood perfusion rate to the brain. The lower permeability of this ester (85b) was probably caused by its excessive lipophilicity beyond an acceptable range for permeability. The rate at which a molecule can be transported to an organ is a function of two factors: the blood flow to the organ and the extraction coefficient of the drug by the organ, which is expressed as  $CL = Q \times E$  where *CL* is the clearance of the drug from the blood in mL/min, *Q* is the blood flow to the target organ in mL/min and *E* is the extraction coefficient (Stella 1980). The extraction coefficient is related to the lipophilicity or *n*-octanol/water partition coefficient of a compound, which is in turn, related to the ability of a compound to partition into phospholipoidal membranes and consequently to organs. Poor transport to the target organ can be due to two factors. First, the physiochemical properties of the drug molecule in question may cause the molecule to be poorly permeable to some rate-limiting membrane such as the BBB. If this is the case, then *E* will be small and *CL* may be largely determined by the extractability of the drug. On the other hand, after intravenous administration, the delivery of lipophilic substances such as 85b to the brain is flow limited, and thus the amount of drug appearing in the brain is proportional to the regional blood flow. After equilibrium is reached, many agents are cleared or washed out from the brain tissue at a rate proportional to regional blood flow. It was reported that for drugs which have *n*-octanol/water partition coefficient values greater than 1.0, their rate of BBB transfer will be limited by the blood perfusion rate in the brain. In contrast, the rate of BBB transfer, for compounds having lower *P* values such as AZT, is limited by the permeability of the BBB. When a drug possesses an intermediate *P* value, its transfer from the blood to the brain will be a function of both permeability and perfusion rate. Lipophilic compounds that have a reversible permeability to the blood-brain barrier (they freely enter and exit) are not optimal for brain delivery due to their rapid clearance. These conclusions are generally limited to small molecules of molecular weights < 450-660 and molecules which do not utilize a carrier-mediated mechanism (Bundgaard, 1985).

(5) This prodrug (85b) may enter certain other tissues such as erythrocytes or fatty tissues, and/or may undergo rapid first-pass bioconversion to zidovudine in the liver or

blood. Binding of the 2-bromoester (85b) to macromolecules may be responsible for decreased extraction of this highly lipophilic compound by the brain. Support for this argument can be found in the work of Hansch and his co-workers, who have reported that the binding of a wide variety of nonionic organic compounds of miscellaneous structure to both bovine serum albumin and bovine hemoglobin appears to depend almost entirely on hydrophobic bonding and is linearly related to their *n*-octanol-water partition coefficients (Dishino *et al.*, 1983).

(6) Due to low lipophilicity, AZT will be trapped in brain long enough to exert its antiviral effects in virus-infected areas of the CNS. There are, however, competing kinetic processes. On one hand, the lipophilic and highly diffusible prodrug 85b could enter and exit brain tissue in response to concentration, but its concentration in brain tissue would also be limited by rapid *in vivo* regeneration to AZT in peripheral tissues. The process of regeneration to AZT in peripheral tissues could result in the production of a highly hydrophilic drug (AZT) which would slowly diffuse back across the BBB in significant levels.

(7) The data might also suggest that the bromoester derivative (85b) is rapidly converted to AZT in mouse brain. In this case a high concentration of AZT should be present in the brain, but this was not observed.

It appears that either diffusion of the prodrug (due to increased lipophilicity) across the BBB is not sufficient to provide an initial high concentration of the prodrug in the brain or that regeneration to AZT in the brain is slow relative to the diffusion across the BBB. Therefore, increased lipophilicity alone does not ensure that a given prodrug will deliver higher concentrations of a parent compound to the CNS. Both the selectivity and absolute rate of bioconversion in the brain are important factors for CNS delivery of conjugates.

The maximum brain AZT concentration was observed 1 min following injection, at the first time point measured. Over the range of time periods studied (1 min to 24 h), the concentration of AZT in the brain declined. This is likely due to bi-directional diffusion of AZT regenerated from the prodrug. The blood AUCs for AZT, when prodrug and AZT were administered were  $12.28 \pm 0.76$  and  $29.12 \pm 2.92$   $\mu\text{mol}\cdot\text{min}/\text{mL}$ , respectively.



However, the brain AUCs for AZT derived from prodrug ( $2.81 \pm 0.5 \mu\text{mol}\cdot\text{min/mL}$ ) was greater than the brain AUC for AZT ( $1.36 \pm 0.023 \mu\text{mol}\cdot\text{min/mL}$ ) when AZT was administered. The time courses for the distribution of AZT in the brain after administration of AZT and the prodrug (85b) are shown in Figure 3.20. As shown in Figure 3.20, the peak AZT level in the brain after ester administration was observed at 1 min. The concentration of AZT detected from the ester 85b was  $25.69 \pm 2.41 \text{ nmol/g}$  in the brain at 1 min is higher than AZT detected following AZT administration ( $9.84 \pm 0.77 \text{ nmol/g}$ ). These concentrations were higher than those previously obtained with the dihydropyridine carrier system coupled to nucleosides (Palomino *et al.*, 1989, Chu *et al.*, 1990) and glycosyl triester derivatives (Namane *et al.*, 1992). For example, Namane *et al.* (1992) measured 1.5 and 4.9 nmol/g of AZT following administration of a glycosyl phosphotriester of AZT and AZT, respectively.

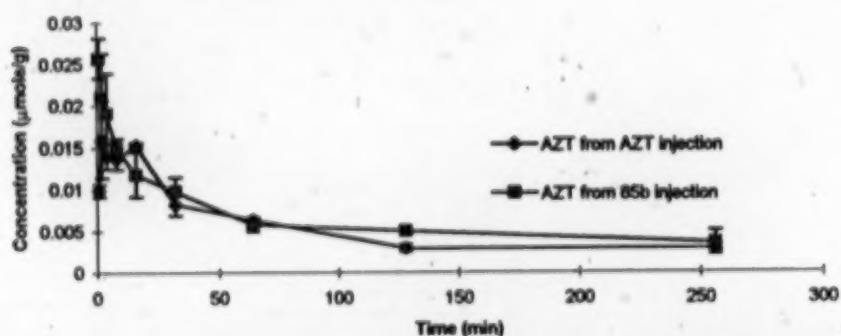


**Figure 3.20.** Concentrations of AZT in brain after iv. tail vein injection of 169.8 mg/kg of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) and 81.5 mg/kg of 2',3'-dideoxy-3'-azidothymidine (6) into mice. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

The relative brain exposure to AZT was calculated as  $r_e = (\text{AUC})_{\text{pd} \rightarrow \text{p}} / (\text{AUC})_{\text{p}} = 2.07$ , where  $(\text{AUC})_{\text{pd} \rightarrow \text{p}}$  equals the area under the AZT brain concentration-time curve following administration of the prodrug (PD, 85b), and  $(\text{AUC})_{\text{p}}$  is the same area obtained following administration of the parent (P,) compound, AZT.  $r_e$  values greater than 1



indicate favorable brain delivery of the parent nucleosides following prodrug administration (Table 3.18). The relative brain exposure for AZT (2.07) indicated a significant increase in exposure to AZT following prodrug (**85b**) administration. The  $r_e$  value clearly demonstrated that AZT had a longer residence time in the brain following prodrug administration than was found following parent drug administration. Experimental studies with this system suggest that the 2-bromoester prodrug to AZT (**85b**) has a pharmacokinetic advantage over AZT (**6**). It was found that systemic administration of the prodrug produced 2.6 times more AZT in the brain than did AZT, 1 min after dosing.

The results for ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine indicate that some molecular redesign may be necessary. Adjustment of the regeneration rate may help to achieve a significantly higher therapeutic concentration of AZT in the brain.

To get a better understanding regarding the final status of **85b**, the concentration of 2-bromomyristic acid (**68c**) in brain following administration of prodrug ester **85b** and fatty acid **68c**, were determined by HPLC using an evaporative light scattering detector (ELSD) and a gradient system. The concentration of 2-bromomyristic acid (**68c**) was not significantly different after injection of fatty acid **68c** or prodrug ester **85b**, since at 2 min, the bromomyristic acid had concentrations of 0.04 and 0.03  $\mu\text{mol/g}$ , respectively. The detection of the fatty acid was not possible at post-injection intervals longer than 2 min due to the limits of detection. The low concentration of fatty acid in brain could be due to rapid metabolism, efflux, high clearance rate from blood, and localization in peripheral tissues such as liver. Compound (**68c**) may partition between many aqueous phases and organic phases when going through the plasma-membrane before it reaches the site of action.

#### 3.3.3.3.1. Brain/blood ratio

This study demonstrated an increase in the ratios of the concentration in the brain as compared to the concentration in blood after prodrug treatment compared with those after AZT administration. Brain/blood concentration ratios for AZT in mice after administration of 50 mg/kg, and 250 mg/kg have been reported to be dose dependent, the ratios being higher after doses of 250 mg/kg (brain/serum =  $0.283 \pm 0.641$ ) than those after doses of

50 mg/kg (brain/serum =  $0.064 \pm 0.025$ ) (Doshi *et al.*, 1989). This ratio was  $0.057 \pm 0.0014$  after an iv bolus dose of 81.5 mg/kg of AZT in the current experiments.

In comparing the areas under the AZT blood and brain concentration curve ( $AUC_{\text{brain}}/AUC_{\text{blood}}$  ratio for AZT), the ratio of values obtained after prodrug (85b) and AZT administration was found to be 0.23 and 0.047, respectively (Table 3.18).

The brain/blood ratio at different times for the AZT concentrations obtained after the administration of either AZT or the prodrugs. The highest brain/blood ratio was observed after AZT dosing at 256 min (0.087), whereas the brain/blood ratio for AZT at 256 min after administration of 85b was found to be 0.23. The brain/blood ratios of 0.12-0.23 were obtained after administration of ester 85b. The data demonstrate that the mean brain/blood drug ratio for AZT released from the prodrug was higher than the ratio for AZT itself (Figure 3.21).

These data indicate that delivery of AZT to the CNS could be significantly increased by prodrug ester delivery of the nucleoside. Both the area under the brain AZT concentration-time curve and the brain: blood ratio were higher after prodrug ester treatment. The absence of the ester in the brain might indicate that the prodrug was partially hydrolyzed immediately after crossing the blood-brain barrier. However, because of the equilibrium between blood and brain concentration, the ester will circulate between these tissues and in each cycle a small amount of ester 85b will hydrolyze to give higher concentration of AZT in the brain.

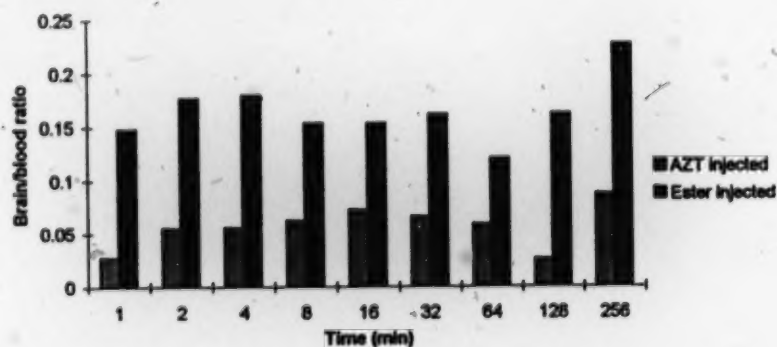


Figure 3.21. Brain-to-blood ratio of AZT after an iv dose of either AZT or AZT-ester.

### 3.4. Summary and Conclusions

Toxicity, rapid blood clearance and limited brain uptake, as well as the appearance of resistance to RT inhibitors, suggest that more potent anti-HIV agents with less toxicity, increased brain delivery and improved pharmacokinetic profiles are needed. A new class of 5'-*O*-myristoyl analogue derivatives of AZT, FLT and dThd have been designed. This research project included the development of new agents with different mechanisms of action as well as improvement of the drugs already shown to be active. Also prodrugs were designed to improve AZT uptake by brain and liver, to slow and prolong release, to minimize toxicity and to improve stability and site specificity.

#### 3.4.1. Myristic acid analogs

(1) Myristic acid analogs including four novel fatty acids, 12-chloro-4-oxadodecanoic acid (65a), 12-azido-4-oxadodecanoic acid (65b), 12-phenoxydodecanoic acid (66i) and 11-(4-iodophenoxy)undecanoic acid (67d) were synthesized. Some myristic acid analogs were shown to have antiviral (66f) and antifungal (68c) properties.

(2) Several ( $\pm$ )-2-halotetradecanoic acids including ( $\pm$ )-2-bromotetradecanoic acid (68c) exhibited potent activity against *C. albicans* (MIC = 39  $\mu$ M), *C. neoformans* (MIC = 20  $\mu$ M), *S. cerevisiae* (MIC = 10  $\mu$ M) and *A. niger* (MIC < 42  $\mu$ M) in RPMI 1640 media.

(3) An optimal *pKa* value between 2.9 and 3.3, and a Log P value between 6.7 and 7.3, appear to be required for potent antifungal activity.

(4) 12-Thioethyldodecanoic acid 66f was moderately active ( $EC_{50}$  = 9.37  $\mu$ M) against HIV-infected CD4 lymphocytes (CEM-SS cell line), and it exhibited *in vitro* activity ( $EC_{50}$  = 17.8  $\mu$ M) against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2).

(5) 12-Methoxydodecanoic acid 66e exhibited *in vitro* activity ( $EC_{50}$  = 20-30  $\mu$ M)

against hepatitis B in the HBV DNA-transfected 2.2.15 cell line.

(6) It appears that there is a correlation between biological activity and Log P or Log D for these fatty acids. Three analogs, 12-thioethyldodecanoic acid (**66f**), 12-bromododecanoic acid (**66d**), and 12-iodododecanoic acid (**66b**), exhibited medium activity against CD4 HIV-infected lymphocytes. These three compounds had partition coefficients between 5.0-5.5. Other compounds with higher or lower partition coefficients were less active.

(7) Replacing one or more methylene groups with oxygen, sulfur, and/or an aromatic ring had a substantial effect on antiviral and antifungal activity. Selectivity with respect to the nature and position of the heteroatom and aromatic groups was observed.

(8) There was a correlation between anti-HIV activity and interatomic distances between carbonyl and heteroatom end terminal moieties of the fatty acid chain. For moderately active compounds (**66b**, **66d** and **66f**), the distances were in the range of 15.16-15.46 Å.

(9) The myristic acid analogs investigated showed differential activity between DHBV and HBV.

(10) Assay results indicate there was no significant inhibition of duck hepatitis B virus (DHBV) replication at a myristic acid analog concentrations of 10 µg/mL.

When taken together, these observations suggest that application of myristic acid analogs may represent a potentially useful strategy for treating viral and fungal infections.

#### 3.4.2. Prodrug esters

(1) A variety of 5'-O-myristoyl analogue derivatives of AZT (**6**), FLT (**7**) and dThd (**8**) were synthesized as new anti-HIV agents and as potential prodrugs to AZT, FLT and

dThd.

(2) 5'-O-(12-Azidododecanoyl)thymidine (81h) exhibited moderate activity ( $EC_{50}$  = 4.6  $\mu$ M) against HIV-infected CD4 lymphocytes. Anti-HIV structure-activity data suggest that the experimental partition coefficient should be in the Log P 4.6-4.8 range for optimal anti-HIV activity of 5'-O-myristoyl thymidine analogs (81a-h).

(3) Modelling studies show that the moderately active 5'-O-ester thymidine derivatives 81d, 81e, and 81h adopt similar C-2' endo sugar conformations and exhibit similar energies for the lowest energy conformer.

(4) There appears to be a strong correlation between anti-HIV activity and Log P for 5'-O-myristoyl analogue derivatives of thymidine, since the contribution of partition coefficient in the QSAR regression equation confirms the importance of this descriptor with respect to anti-HIV-1 activity for these esters (81a-h).

(5) It was concluded that the ester prodrug approach described could serve as a useful method to improve the *in vivo* characteristics of anti-HIV and anti-HBV nucleosides. The analogs are less, equal or more potent anti-HIV agents than their corresponding parent compounds *in vitro*, depending upon the substituents at the 5'-position.

(6) Most 5'-substituents AZT derivatives, i.e. 82a, 82b, 83a, 83c, 83f, 83g, 84b, 84d, 84e and 85b decreased anti-HIV activity. However, several 5'-substituted AZT derivatives, i.e. 82c, 82e, 83d, 84a, 84c, and 87 demonstrated anti-HIV activity that was equivalent or more that of AZT.

(7) Some prodrugs of AZT (82c, 82e, 83d and 87) were found to be the most effective anti-HIV-1 agents. It was found that three AZT prodrugs, 3'-azido-2',3'-deoxy-5'-O-(4-oxatetradecanoyl)thymidine (82c) ( $EC_{50}$  = 1.4 nM), 3'-azido-2',3'-deoxy-5'-O-(12-bromododecanoyl)thymidine (83d) ( $EC_{50}$  = 3.2 nM) and 3'-azido-2',3'-deoxy-5'-O-



(tetradecanoyl)thymidine (87) ( $EC_{50} = 3.8 \text{ nM}$ ) are the most effective anti-HIV-1 agents. The high selectivity of these analogs makes them important compounds in view of their *in vivo* anti-HIV activity. It's concluded that the placement of oxygen in the 4-position (82c) and the placement of bromine in the 12-position (83d) are important requirements for potency and selectivity requirements of these analogs.

(8) 3'-Azido-2',3'-dideoxy-5'-O-(12-methoxydodecanoyl)thymidine (83e) showed high activity ( $EC_{50} = 2.7 \pm 0.3 \text{ }\mu\text{M}$ ) and low toxicity ( $IC_{50} = 727 \pm 19 \text{ }\mu\text{M}$ ) against HBV replication in 2.2.15 cells. This group of prodrugs may provide a high concentration of the parent nucleoside within a short time period or act as a sustained releaser of the parent drug in liver.

(9) This study shows that the lipophilicities of prodrugs are superior to those of the parent drug. This property could permit better penetration of the drug through biological membranes. Their enhanced lipophilicity ( $\text{Log } P = 4.50\text{--}8.14$  range) relative to the parent compounds AZT ( $\text{Log } P = 0.06$ ) and FLT ( $\text{Log } P = -0.52$ ), may enhance their ability to cross the BBB to provide a higher concentration of active drug in the brain.

(10) There is a linear correlation between the  $\text{Log } P$  and  $\text{Log}$  (retention times) for 5'-O-AZT, FLT and dThd esters.

(11) The *in vitro* enzymatic hydrolysis of esters (81a-93) by porcine liver esterase, rat plasma and rat brain homogenate was studied. Hydrolysis of the ester analogs was found to be dependent upon the nature of the substituent at the 5'-position, the nucleoside, and steric and medium effects. It may be possible to control the rate of AZT release *in vivo*, which would influence the blood half-life and toxicity, by selecting the appropriate substituent on a fatty acid chain. A relatively high cleavage of the ester mostly in plasma was observed.

(12) An increase in the steric bulk of the 5'-O-myristoyl substituent in compounds such

as **85b** decreased hydrolysis rate and appeared to inhibit binding of the substrate to the active center of the enzyme.

(13) Careful selection of appropriate ester substituents provided a prodrug (**85b**) which would undergo very rapid bioactivation reactions to provide a high concentration of AZT at the desired site of actions.

(14) The disposition of Zidovudine (**6**) and ester (**85b**) *in vivo* was best described by a two-compartment model.

(15) The *in vivo* distribution study of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomystoyl)thymidine (**85b**) after *iv* injection via the tail vein was carried out in conventional Balb/c mice. Based on the results from the *in vitro* and *in vivo* studies, it was demonstrated that the 5'-O-ester model improved the pharmacokinetic parameters and hepatic and brain delivery of AZT.

(16) The data indicate that lipophilic prodrugs of these antiviral nucleosides might increase the *in vivo* efficacy and selectivity of nucleoside therapy for hepatitis B infection by delivering a much larger fraction of the administered drug to the liver (**85b**). These experiments have provided very interesting findings which include an increased concentration of AZT in the liver and prolonged liver levels of the 2-bromoester (**85b**).

(17) The prodrug (**85b**) provided a higher brain concentration of AZT with a significantly improved pharmacokinetic profile of AZT in the brain compared to that after AZT administration (peak concentration of 25.7 nmol/g vs 9.8 nmol/g; AUC 2.81  $\mu\text{mol}\cdot\text{min/g}$  vs. 1.36  $\mu\text{mol}\cdot\text{min/g}$ ).

(18) The biodistribution study did show an improvement with respect to the AZT brain targeting and AZT residence time in blood. Mean brain/blood concentration ratios for AZT after prodrug ester administration were greater than those after AZT injection. The

highest brain/blood ratios of 0.087 and 0.23 were observed for AZT and prodrug ester (85b), respectively at 256 min postdosing.

### 3.5. Future directions

(1) The increasing lipophilic nature of the ester prodrugs as demonstrated by their high partition coefficient (Log P) was expected to increase the likelihood of their being used as potential candidates for chylomicron-assisted lymphatic transport. The 2-bromoester (85b) will probably be absorbed mostly by the lymphatic system which may allow lymphatic system to be targeted for the treatment of infection. Further studies are required to study movement of 85b in the lymphatic system.

(2) Study of the transcutaneous absorption of lipophilic derivatives is important since drugs applied to the skin are poorly absorbed. Drug absorption from skin can be improved by increasing lipophilicity. According to Seki *et al.* (1990), among the aliphatic esters of AZT, acetate (C2-AZT) and hexanoate (C6-AZT) showed 2.4- and 4.8-fold enhanced permeation to human skin relative to application of AZT itself, respectively. The 5'-*O*-myristoyl analogue derivatives of AZT and FLT were lipophilic, so their penetration through the lipid matrix of the stratum corneum and also their penetration into cells might be more efficient. More research is required to confirm this postulation.

(3) Assessing the mechanism of myristic acid analog uptake into lymphocytes and the pathway for their subsequent metabolic processing is required to develop systems that will more accurately forecast the ability of different classes of myristic acid analogs to inhibit HIV-I replication. The utility of myristic acid analogs as therapeutic agents will be enhanced by a clearer understanding of the mechanisms for their selective uptake, metabolism, and incorporation, as well as an appreciation of the contribution of the myristoyl moiety to the function of a specific targeted *N*-myristoylprotein.

(4) The antiviral activity results can be used to design a more selective and more potent analog like 3'-azido-2',3'-dideoxy-5'-*O*-(12-bromo-4-oxadodecanoyl)thymidine

that has both functional groups present in 82c and 83d.

(5) The enhanced therapeutic activity of conjugates 82c, 82e and 83d warrants further clinical investigation. Further metabolism studies are required in CEM cells to determine the mechanism of their antiviral activity.

## 4. EXPERIMENTAL SECTION

### 4.1. Material and methods

Thymidine (dThd, 8), 12-bromododecanoic acid (66d), 12-hydroxydodecanoic acid (66j), 11-bromoundecanoic acid (67c), 11-aminoundecanoic acid (67f), ( $\pm$ )-2-hydroxytetradecanoic acid (68b), ( $\pm$ )-2-bromotetradecanoic acid (68c), myristic acid (70), *n*-octanol and all other reagents and chemicals were purchased from the Aldrich Chemical Co, unless noted otherwise. *n*-Octanol (99%, Aldrich Chemical Company, Inc.), used for measurement of P values, was distilled before use. Phosphate buffer solution (0.2 M, pH = 7.4) used for measurement of P values prepared. All solvents used for HPLC analyses were of HPLC grade that were purchased from Mallinckrodt and degassed before use. Benzene and dimethyl formamide (DMF) were dried by refluxing in the presence of calcium hydride. All solvents used were analytical grade.

RPMI 1640 media, obtained from the GIBCO BRL Co., was buffered with 0.165 M 3-[*N*-morpholino]propane-sulfonic acid (MOPS) and sodium hydroxide to adjust the pH to 7.0 at 25 °C.

Porcine liver esterase (EC 3.1.1.1) suspension in 3.2 M  $(\text{NH}_4)_2\text{SO}_4$  (200 units/mg protein) was purchased from the Sigma Chemical Co.

2',3'-Dideoxy-3'-thiacytidine (3TC, 4), 2',3'-dideoxycytidine (ddC, 5), 3'-azidodeoxythymidine (AZT, 6) and 3'-fluoro-3'-deoxythymidine (FLT, 7) were used as the standard compounds in different experiments.

### 4.2. Animals

Male Sprague-Dawley rats (250-280 g) and male Balb/c mice (25-30 g) were purchased from the University of Alberta Animal Services Facility. All animals were housed in the Dentistry-Pharmacy Building Animal Services Facility and fed conventional rodent foods. Mice were housed up to five per cage and rats up to three per cage. All studies were performed according to the Canadian Council on Animal Care guidelines, with review and approval of the experimental protocols by the University of Alberta Health Sciences Animal Welfare Committee.



#### 4.3. Physical constants and spectroscopy

Melting points were determined with a Büchi capillary apparatus and are uncorrected. Nuclear magnetic resonance spectra ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR,  $^{19}\text{F}$  NMR) were determined on a Bruker AM-300 spectrometer. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of  $\text{D}_2\text{O}$ .  $^{13}\text{C}$  NMR spectra were acquired using the J modulated spin echo technique where methyl and methine carbon resonances appear as positive peaks, and methylene and quaternary carbon resonances appear as negative peaks. High resolution mass spectra were determined on an AEI MS 50 spectrometer equipped with a Mass Spectrometry Services MASPEC data system. Quantitative ultraviolet (UV) absorption spectra were recorded on a Shimadzu UV 160 spectrophotometer. IR spectra were recorded using a Nicolet 5DX-FT spectrometer. Microanalyses, which were performed by the Chemistry Department, University of Alberta, using an EA1108-Elemental Analyzer (Carlo Erba Instruments), were within  $\pm 0.4\%$  of theoretical values for all elements listed, unless otherwise indicated.

#### 4.4. Other equipment

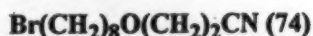
A mechanical shaker (Dubnoff metabolic shaking incubator, Precision Scientific Co.) was used for partition coefficient and stability determinations. Eppendorf micropipettes were used for measurement of micro quantity of liquids. All centrifugations were performed using an Eppendorf 5412 microcentrifuge and Eppendorf microcentrifuge tubes unless otherwise specified. A Vortex-Genie (Scientific Industries, Inc. Bohemia, N. Y. 11716, USA) was used for mixing small samples.

## 4.5. Synthetic chemistry

### 4.5.1. Fatty Acids

#### 4.5.1.1. 4-Oxaderivatives

##### 4.5.1.1.1 12-Bromo-4-oxadodecanonitrile (74)



Sodium hydride (5.92 mg, 0.25 mmol, 97%) was added to a solution of 8-bromo-1-octanol (72, 1.00 g, 0.82 mL, 4.78 mmol), and the mixture was stirred for 0.5 h at room temperature. Acrylonitrile (73, 0.80 mL, 12.05 mmol) was added slowly to this mixture during 5 min at 55-60 °C. After stirring for 3 h at 55-60 °C, and then for 15 h at room temperature, ether (24 mL) was added. The organic phase was washed with water (8 mL), brine (8 mL) and dried ( $\text{Na}_2\text{SO}_4$ ). The residue, which consisted of one major product, was purified by silica gel column chromatography using hexane-ethyl acetate, 80:20 (v/v) as eluent to yield 12-bromo-4-oxadodecanonitrile (74) as a liquid (1.1 g, 85%) which was used immediately for the synthesis of 12-chloro-4-oxadodecanoic acid (65a).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.22-1.48 (br m, 8H, methylene envelope), 1.54 (m,  $J = 6.4$  Hz,  $J = 6.5$  Hz, 4H,  $\text{OCH}_2\text{CH}_2$ ), 1.83 (quint,  $J = 6.5$  Hz,  $J = 7.2$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 2.67 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{CN}$ ), 3.48 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{Br}$ ), 3.54 (t,  $J = 6.4$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.61 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  18.17, 25.72, 27.88, 28.47, 28.99, 29.26, 32.60, 33.84 (methylene carbons), 65.11 ( $\text{CH}_2\text{O}$ ), 71.24 ( $\text{CH}_2\text{O}$ ), 117.79 (CN).

##### 4.5.1.1.2. 12-Chloro-4-oxadodecanoic acid (65a)



A solution of the nitrile (74, 1.06 g, 4.05 mmol) in acetic acid (5 mL) and 37% (w/v) hydrochloric acid (15 mL) was refluxed for 2 h. After removal of the solvent *in vacuo*, water (50 mL), and then ethyl acetate (150 mL) were added to the residue. The organic phase was washed with water (50 mL), brine (50 mL), and dried ( $\text{Na}_2\text{SO}_4$ ).

The residual oil obtained was purified by Kugelrohr distillation to afford **65a** (0.72 g, 76%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.22-1.59 (br m, 8H, methylene envelope), 1.36-1.59 (m, 2H,  $\text{CH}_2$ ), 1.69 (quint,  $J = 7.0$  Hz,  $J = 6.5$  Hz, 2H,  $\text{CH}_2$ ), 2.55 (t,  $J = 6.5$  Hz, 3H,  $\text{CH}_2\text{CO}$ ), 3.38 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.45 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{Cl}$ ), 3.63 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 11.05 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  25.75, 26.62, 28.63, 29.04, 29.23, 32.45 (methylene carbons), 34.74 ( $\text{CH}_2\text{CO}$ ), 44.92 ( $\text{CH}_2\text{Cl}$ ), 65.54 ( $\text{CH}_2\text{O}$ ), 71.04 ( $\text{CH}_2\text{O}$ ), 177.32 ( $\text{COO}$ ).

*Anal.* Calcd. for  $\text{C}_{11}\text{H}_{21}\text{ClO}_3$ : C 55.81, H 8.94; found C 56.19, H 8.97.

#### 4.5.1.1.3. 12-Azido-4-oxadodecanoic acid (**65b**)



A solution of the 12-chloro-4-oxadodecanoic acid (**65a**, 499.0 mg, 2.10 mmol) and sodium azide (395.2 mg, 6.1 mmol) in DMF (8.5 mL) was stirred at  $60^\circ\text{C}$  for 8 h. After removal of the solvent *in vacuo*, the residue was acidified with hydrochloric acid (8.2 mL, 1N). The solution was extracted with dichloromethane ( $2 \times 25$  mL). The organic phase was washed with water (20 mL), brine (20 mL), and dried ( $\text{Na}_2\text{SO}_4$ ). The organic solvent was evaporated under reduced pressure and the residual oil obtained was purified by Kugelrohr distillation to afford **65b** (184 mg, 36%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.20-1.46 (br m, 8H, methylene envelope), 1.50-1.70 (m, 4H,  $\text{N}_3\text{CH}_2\text{CH}_2$ ,  $\text{OCH}_2\text{CH}_2$ ), 2.64 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.26 (t,  $J = 7.0$  Hz, 2H,  $\text{CH}_2\text{N}_3$ ), 3.46 (t,  $J = 6.7$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.71 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 10.2 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  25.88, 26.59, 28.76, 29.01, 29.17, 29.39 (methylene carbons), 34.83 ( $\text{CH}_2\text{CO}$ ), 51.42 ( $\text{CH}_2\text{N}_3$ ), 65.55 ( $\text{CH}_2\text{O}$ ), 71.22 ( $\text{CH}_2\text{O}$ ), 176.93 ( $\text{COO}$ ).

*Anal.* Calcd for  $\text{C}_{11}\text{H}_{21}\text{N}_3\text{O}_3$ : C 54.30, H 8.70, N 17.27; found C 54.68, H 9.05, N 16.84.

#### 4.5.1.2. 12-Substituted analogs

##### 4.5.1.2.1. Methyl 12-hydroxydodecanoate (75)

###### $\text{HO}(\text{CH}_2)_{11}\text{COOMe}$ (75)

Chlorotrimethylsilane (0.81 g, 0.95 mL, 7.45 mmol) was added to a solution of 12-hydroxydodecanoic acid (66j, 0.50 g, 2.31 mmol) in dry methanol (12.0 mL) under a nitrogen atmosphere and the reaction mixture was stirred at room temperature for 16 h. Methanol was removed *in vacuo* and the residue obtained was recrystallized from petroleum ether to afford 10 (0.48 g, 89 %) which was used immediately for the synthesis of methyl 12-fluorododecanoate (11); mp 31-32 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.17-1.3 (br m, 14H, methylene envelope), 1.36-1.56 (br m, 4H,  $\text{CH}_2\text{CH}_2\text{OH}$ ,  $\text{CH}_2\text{CH}_2\text{COOMe}$ ), 2.27 (t,  $J = 7.7$  Hz, 2H,  $\text{CH}_2\text{COOMe}$ ), 2.30 (s, 1H, OH), 3.60 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{OH}$ ), 3.63 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  24.88, 25.66, 28.58, 29.05, 29.14, 29.40, 29.46, 32.68 (methylene carbons), 34.05 ( $\text{CH}_2\text{CO}$ ), 51.36 ( $\text{OCH}_3$ ), 62.94 ( $\text{CH}_2\text{OH}$ ), 174.32 ( $\text{COO}$ ).

##### 4.5.1.2.2. Methyl 12-fluorododecanoate (76)

###### $\text{F}(\text{CH}_2)_{11}\text{COOMe}$ (76)

Diethylaminosulfur trifluoride (DAST) (0.61 g, 0.5 mL, 3.78 mmol) was added to a solution of methyl 12-hydroxydodecanoate (75, 0.23 g, 1 mmol) in 20 mL anhydrous benzene and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was mixed with 5% sodium bicarbonate (20 mL) and extracted with ethyl acetate ( $3 \times 50$  mL). The organic phase was separated, washed with water, dried ( $\text{Na}_2\text{SO}_4$ ) and the solvent was removed *in vacuo*. The residue, which consisted of one major product, was purified by silica gel column chromatography using hexane-ethyl acetate, 70:30 (v/v) as eluent to yield methyl 12-fluorododecanoate (76) as a liquid (0.2 g, 86 %) which was used immediately for the synthesis of 12-fluorododecanoic acid (66a).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.17-1.37 (br m, 14H, methylene

envelope), 1.47-1.71 (br m, 4H,  $\text{CH}_2\text{CH}_2\text{F}$ ,  $\text{CH}_2\text{CH}_2\text{COOMe}$ ), 2.23 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{COOMe}$ ), 3.58 (s, 3H,  $\text{OCH}_3$ ), 4.34 (dt,  $J_{\text{H,F}} = 47.5$  Hz, 2H,  $J_{\text{CH}_2,\text{CH}_2} = 6.3$  Hz,  $\text{CH}_2\text{F}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  24.82, 24.98, 25.06, 29.00, 29.10, 29.26, 29.33 (methylene carbons), 33.93 ( $\text{CH}_2\text{CO}$ ), 51.20 ( $\text{OCH}_3$ ), 83.95 (d,  $J_{\text{C,F}} = 163.39$  Hz,  $\text{CH}_2\text{F}$ ), 174.08 ( $\text{COO}$ ).

#### 4.5.1.2.3. 12-Fluorododecanoic acid (66a)

##### $\text{F}(\text{CH}_2)_{11}\text{COOH}$ (66a)

A mixture of methyl 12-fluorododecanoate (76, 0.20 g, 0.86 mmol) in dioxane (0.27 mL) and 37% (w/v) hydrochloric acid (0.45 mL) was refluxed for 2 h. After cooling to room temperature, the mixture was extracted with chloroform ( $3 \times 1$  mL) and the combined chloroform solutions were extracted with 10% (w/v) aqueous potassium hydroxide solution (5 mL). The combined alkaline solutions were washed with diethyl ether ( $3 \times 1$  mL) and acidified with concentrated HCl. The carboxylic acid which separated was taken up in chloroform (10 mL) and the chloroform solution was dried ( $\text{Na}_2\text{SO}_4$ ). The solvent was removed *in vacuo* and the residue obtained, which consisted of one major product, was purified by silica gel column chromatography using hexane-ethyl acetate, 80:20 (v/v) as eluent. Recrystallization from petroleum ether yielded 12-fluorododecanoic acid (66a, 0.12 g, 65%); mp 59-60 °C (*ref.* (Hudlicky *et al.*, 1988) 59.5-61 °C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.10-1.40 (br m, 14H, methylene envelope), 1.46-1.80 (br m, 4H,  $\text{CH}_2\text{CH}_2\text{F}$ ,  $\text{CH}_2\text{CH}_2\text{COOH}$ ), 2.28 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{COOH}$ ), 4.36 (dt,  $J_{\text{H,F}} = 47.4$  Hz,  $J_{\text{CH}_2,\text{CH}_2} = 6.3$  Hz, 2H,  $\text{CH}_2\text{F}$ ), 10.00 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  24.61, 25.06, 25.12, 28.98, 29.16, 29.31, 29.40, 30.23, 30.48 (methylene carbons), 33.96 ( $\text{CH}_2\text{CO}$ ), 84.17 (d,  $J_{\text{C,F}} = 164.7$  Hz,  $\text{CH}_2\text{F}$ ), 179.77 ( $\text{COO}$ );  $^{19}\text{F}$  NMR ( $\text{CDCl}_3$ )  $\delta$  56.79 (dddd,  $J_{\text{gem}} = 47.4$  Hz,  $J_{\text{vic}} = 24.4$  Hz, 1F,  $\text{CH}_2\text{F}$ ).



## 4.5.1.2.4. 12-Iodododecanoic acid (66b)



A mixture of 12-bromododecanoic acid (66d, 0.25 g, 0.9 mmol) and sodium iodide (0.40 g, 2.7 mmol) in dry acetone (7.0 mL) was stirred for 18 h at room temperature. The solvent was removed *in vacuo*, the residue obtained was dissolved in water (60 mL) and extracted with chloroform (3 × 60 mL). The combined extracts were dried ( $\text{Na}_2\text{SO}_4$ ), and the solvent was removed *in vacuo*. The residue obtained was recrystallized from petroleum ether to afford 12b (0.28 g, 95%); mp 62-63 °C (*ref.* (Kruizinga *et al.*, 1981) 61-62.5 °C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.14-1.44 (br m, 14H, methylene envelope), 1.54 (quint,  $J = 7.7$  Hz,  $J = 7.1$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{COOH}$ ), 1.74 (quint,  $J = 7.1$  Hz,  $J = 6.6$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{I}$ ), 2.36 (t,  $J = 7.1$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.20 (t,  $J = 7.1$  Hz, 2H,  $\text{CH}_2\text{I}$ ), 10.3 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.19 ( $\text{CH}_2\text{I}$ ), 24.63, 28.51, 28.68, 29.02, 29.17, 29.34, 29.40, 30.47, 33.56 (methylene carbons), 34.05 ( $\text{CH}_2\text{CO}$ ), 180.17 ( $\text{COO}$ ).

*Anal.* Calcd. for  $\text{C}_{12}\text{H}_{23}\text{IO}_2$ : C 44.18, H 7.11; found C 44.34, H 7.29.

## 4.5.1.2.5. 12-Chlorododecanoic acid (66c)



Nitric acid (70% w/v, 2.2 mL) was added to a mixture of hydrochloric acid (37% w/v, 1.5 mL) and 12-iodododecanoic acid (66b, 0.20 g, 0.61 mmol) at 15-20 °C. The reaction was allowed to proceed at 15-20 °C with stirring for 2 h, the precipitated elemental iodine was filtered off and the filtrate was poured onto ice (1.0 g). The aqueous layer was extracted with methylene chloride (3 × 3 mL), the methylene chloride extract was washed consecutively with water, a 2% (w/v) aqueous  $\text{Na}_2\text{S}_2\text{O}_3$  to remove iodine and with water. The organic phase was dried ( $\text{Na}_2\text{SO}_4$ ) and the solvent was removed *in vacuo* and the residue obtained, which consisted of one major product, was purified by silica gel column chromatography using hexane-ethyl acetate,

80:20 (v/v) as eluent to yield **66e** (96 mg, 66%); (mp 42.5–43 °C) (*ref.* (Bidd *et al.*, 1987) 43–44 °C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.18–1.46 (br m, 14H, methylene envelope), 1.52 (quint, 2H,  $\text{CH}_2$ ), 1.73 (quint, 2H,  $\text{CH}_2$ ), 2.32 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.50 (t,  $J = 6.9$  Hz, 2H,  $\text{CH}_2\text{Cl}$ ), 10.94 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  24.64, 26.86, 29.85, 29.01, 29.18, 29.35, 29.40, 32.64 (methylene carbons), 34.02 ( $\text{CH}_2\text{CO}$ ), 45.15 ( $\text{CH}_2\text{Cl}$ ), 180.10 ( $\text{COO}$ ).

*Anal.* Calcd. for  $\text{C}_{12}\text{H}_{23}\text{ClO}_2 \cdot 1/4 \text{H}_2\text{O}$ : C 60.24, H 9.90; found C 60.02, H 9.69.

#### 4.5.1.2.6. 12-Phenoxydodecanoic acid (**66i**)

##### $\text{PhO}(\text{CH}_2)_{11}\text{COOH}$ (**66i**)

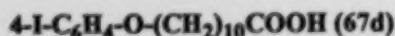
Phenol (0.17 g, 1.81 mmol) was dissolved in ethanol (19 mL) and potassium hydroxide (0.23 g, 4.10 mmol) was added. 12-Bromododecanoic acid (**66d**, 0.50 g, 1.79 mmol) was dissolved in ethanol (10 mL) and added to the first solution under a nitrogen atmosphere. The reaction mixture was refluxed under nitrogen for 4 h, acidified with a solution of 37% (w/v) hydrochloric acid (10 mL) in water (100 mL) and the solvents were removed *in vacuo*. The residue was dissolved in water (50 mL), extracted with chloroform ( $3 \times 60$  mL), the organic phase was dried ( $\text{Na}_2\text{SO}_4$ ) and the solvent was removed *in vacuo*. The residue obtained was recrystallized from petroleum ether-ethyl acetate to give crystals which consisted of one major product that was purified by silica gel column chromatography using hexane-ethyl acetate, 80:20 (v/v) as eluent to yield 12-phenoxydodecanoic acid (**66i**) which was recrystallized from hexane (0.5 g, 96%); mp 76–77 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.66 (quint,  $J = 7.5$  Hz,  $J = 7.2$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.81 (quint,  $J = 7.0$  Hz,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.22–1.54 (br m, 14H, methylene envelope), 2.37 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.88 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 6.86–7.00 (m, 3H, aromatic), 7.23–7.60 (m, 2H, aromatic), 10.30 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  24.62, 25.63, 26.00, 28.99, 29.17, 29.26, 29.34, 29.44 (methylene carbons), 34.05 ( $\text{CH}_2\text{CO}$ ), 67.83 ( $\text{CH}_2\text{O}$ ), 114.48 (aromatic C), 120.40 (aromatic C), 129.33 (aromatic C), 159.10

(aromatic C), 180.22 (COO).

*Anal.* Calcd. for  $C_{18}H_{28}O_3 \cdot 1/2 H_2O$ : C 71.73, H 9.70; found C 71.77, H 9.54.

#### 4.5.1.3. 11-Substituted analogs

##### 4.5.1.3.1. 11-(4-Iodophenoxy)undecanoic acid (67d)

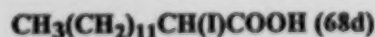


The same procedure used for the synthesis of 66i, but using 4-iodophenol in the place of phenol and 11-bromoundecanoic acid (67c) in place of 12-bromododecanoic acid (66d) afforded 67d (0.15 g, 43%); mp 90-91 °C;  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.22-1.50 (br m, 12H, methylene envelope), 1.63 (quint,  $J = 7.2$  Hz,  $J = 7.2$  Hz, 2H,  $CH_2$ ), 1.76 (quint,  $J = 7.2$  Hz,  $J = 6.6$  Hz, 2H,  $CH_2$ ), 2.35 (t,  $J = 7.2$  Hz, 2H,  $CH_2CO$ ), 3.90 (t,  $J = 7.2$  Hz, 2H,  $CH_2O$ ), 6.66 (m,  $J_{ortho} = 8.8$  Hz,  $J_{meta} = 3.5$  Hz, 2H, aromatic), 7.53 (m,  $J_{ortho} = 8.8$  Hz,  $J_{meta} = 3.5$  Hz, 2H, aromatic), 9.25 (1H, br s, COOH);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  24.64, 25.96, 29.01, 29.11, 29.16, 29.29, 29.43 (methylene carbons), 33.99 ( $CH_2CO$ ), 68.11 ( $CH_2O$ ), 82.39 (aromatic C), 116.96 (aromatic C), 138.15 (aromatic C), 159.03 (aromatic C), 179.85 (COO).

*Anal.* Calcd. for  $C_{17}H_{25}IO_3$ : C 50.51, H 6.23; found C 50.99, H 6.36.

#### 4.5.1.4. 2-Substituted analogs

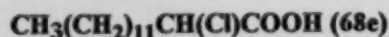
##### 4.5.1.4.1. ( $\pm$ )-2-Iodotetradecanoic acid (68d)



A mixture of ( $\pm$ )-2-bromotetradecanoic acid (68c, 0.50 g, 1.63 mmol) and sodium iodide (0.729 g, 4.86 mmol) in dry acetone (12.7 mL) was stirred for 18 h at room temperature. The solvent was evaporated *in vacuo*, the residue was dissolved in water (60 mL) and extracted with chloroform (3  $\times$  60 mL). The combined extracts were dried ( $Na_2SO_4$ ), filtered, and the solvent was removed *in vacuo*. The residue was recrystallized from petroleum ether to afford 68d (0.48 g, 83%); mp 47-48 °C;  $^1H$

NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (t,  $J = 7.0$  Hz, 3H,  $\text{CH}_3$ ), 1.2-1.5 (br m, 20H, methylene envelope), 1.92-2.4 (m, 2H,  $\text{CH}_2\text{CHICOOH}$ ), 4.31 (t,  $J = 7.5$  Hz, 2H,  $\text{CHI}$ ), 9.3 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.10 ( $\text{CH}_3$ ), 20.13 ( $\text{CHI}$ ), 22.67, 28.69, 29.31, 29.48, 29.58, 29.63, 31.91 (methylene carbons), 35.81 ( $\text{CH}_2\text{CHI}$ ), 177.35 ( $\text{COOH}$ ); MS (70 eV)  $m/z$  (%) = 354 (3) [ $\text{M}^+$ ], 227 (57) [ $\text{M}^+ - \text{I}^-$ ], 209 (22) [ $\text{M}^+ - \text{H}_2\text{O} - \text{I}^-$ ], 57 (89) [ $\text{C}_4\text{H}_9^+$ ], 55 (100) [ $\text{C}_4\text{H}_7^+$ ].

#### 4.5.1.4.2. ( $\pm$ )-2-Chlorotetradecanoic acid (68e)



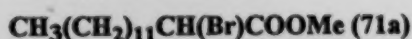
Nitric acid (70% w/v, 4.11 mL) was added to a mixture of 37% (w/v) hydrochloric acid (2.8 mL) and ( $\pm$ )-2-iodotetradecanoic acid (68d) (0.411 g, 1.16 mmol) at 15-20 °C and the mixture was stirred at this temperature for 2 h. The precipitated solid, which was filtered off, consisted of one major product that was purified by silica gel column chromatography using hexane-ethyl acetate (70:30, v/v) as eluent to yield 68e (87 mg, 29%); mp 43-44 °C (*ref.* Ogata *et al.*, 1979) mp 41-42 °C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.90 (t,  $J = 7.0$  Hz, 3H,  $\text{CH}_3$ ), 1.2-1.6 (br m, 20H, methylene envelope), 1.90-2.16 (m, 2H,  $\text{CH}_2\text{CHClCOOH}$ ), 4.33 (dd,  $J = 6.0$  Hz,  $J = 8.1$  Hz, 2H,  $\text{CHCl}$ ), 10.0 (br s, 1H,  $\text{COOH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.09 ( $\text{CH}_3$ ), 22.67, 25.88, 28.81, 29.29, 29.46, 29.61, 31.91 (methylene carbons), 34.77 ( $\text{CH}_2\text{CHCl}$ ), 57.09 ( $\text{CHCl}$ ), 174.99 ( $\text{COOH}$ ).

#### 4.5.1.4.3. ( $\pm$ )-2-Bromotetradecanoate esters (71a-c): General synthesis

Chlorotrimethylsilane (0.47 g, 0.52 mL, 4.32 mmol) was added to a solution of ( $\pm$ )-2-bromotetradecanoic acid (68c, 0.392 g, 1.28 mmol) in dry alcohol (6.62 mL) under a nitrogen atmosphere and the mixture was stirred at room temperature for 16 h. The solvent was removed under reduced pressure and the residue obtained consisted of one major product that was purified by silica gel column chromatography

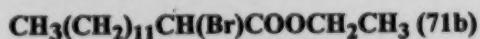
using hexane-ethyl acetate (70:30, v/v) as eluent to yield the ester product as a liquid.

#### 4.5.1.4.3.1. (±)-Methyl 2-bromotetradecanoate (71a)



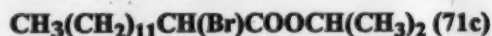
The method described above was carried out using dry methanol to yield 71a (377 mg, 92%) as an oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.85 (t,  $J = 7.0$  Hz, 3H,  $\text{CH}_3$ ), 1.16-1.50 (br m, 20H, methylene envelope), 1.88-2.12 (br m, 2H,  $\text{CH}_2\text{CHBr}$ ), 3.75 (s, 3H,  $\text{OCH}_3$ ), 4.18 (t,  $J = 7.3$  Hz, 1H,  $\text{CHBr}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.03 ( $\text{CH}_3$ ), 22.61, 27.20, 28.76, 29.27, 29.41, 29.53, 29.57, 31.86 (methylene carbons), 34.88 ( $\text{CH}_2\text{CHBr}$ ), 45.64 ( $\text{CHBr}$ ), 52.73 ( $\text{OCH}_3$ ), 170.27 ( $\text{COO}$ ); MS (70 eV)  $m/z$  (%) = 321 (10) [ $\text{M}^+$ ], 241 (64) [ $\text{M}^+ - \text{Br}^-$ ], 87 (100) [ $\text{C}_4\text{H}_7\text{O}_2^+$ ], 55 (62) [ $\text{C}_4\text{H}_7^+$ ].

#### 4.5.1.4.3.2. (±)-Ethyl 2-bromotetradecanoate (71b)

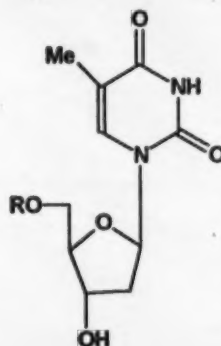


The general synthetic method described above using dry ethanol afforded 71b (307 mg, 72%) as an oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  0.87 (t,  $J = 6.8$  Hz, 3H,  $\text{CH}_3$ ), 1.18-1.52 (br m, 23H, methylene envelope,  $\text{OCH}_2\text{CH}_3$ ), 1.88-2.14 (br m, 2H,  $\text{CH}_2\text{CHBr}$ ), 4.21 (t,  $J = 7.3$  Hz, 1H,  $\text{CHBr}$ ), 4.21 (q,  $J = 7.3$  Hz, 2H,  $\text{OCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  13.92 ( $\text{CH}_3$ ), 14.06 ( $\text{CH}_3$ ), 22.64, 27.21, 28.79, 29.28, 29.43, 29.59, 31.88 (methylene carbons), 34.86 ( $\text{CH}_2\text{CHBr}$ ), 46.15 ( $\text{CHBr}$ ), 61.80 ( $\text{OCH}_3$ ), 169.86 ( $\text{COO}$ ); MS (70 eV)  $m/z$  (%) = 335 (11) [ $\text{M}^+$ ], 255 (71) [ $\text{M}^+ - \text{Br}^-$ ], 237 (7) [ $\text{M}^+ - \text{H}_2\text{O} - \text{Br}^-$ ], 101 (54) [ $\text{C}_5\text{H}_9\text{O}_2^+$ ], 69 (50) [ $\text{C}_5\text{H}_9^+$ ], 55 (100) [ $\text{C}_4\text{H}_7^+$ ].



4.5.1.4.3.3. ( $\pm$ )-Isopropyl 2-bromotetradecanoate (71c)

The same procedure using dry isopropyl alcohol gave 71c (407 mg, 92%) as an oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (t,  $J$  = 6.0 Hz, 3H,  $\text{CH}_3$ ), 1.16-1.52 (br m, 26H, methylene envelope  $\text{OCH}(\text{CH}_3)_2$ ), 1.88-2.13 (br m, 2H,  $\text{CH}_2\text{CHBr}$ ), 4.16 (t,  $J$  = 7.3 Hz, 3H,  $\text{CHBr}$ ), 5.06 (sept,  $J$  = 6.0 Hz, 1H,  $\text{OCH}(\text{CH}_3)_2$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.95 ( $\text{CH}_3$ ), 14.05 ( $\text{CH}_3$ ), 21.32, 21.49, 21.80, 22.64, 27.21, 28.79, 29.28, 29.43, 31.58, 31.87 (methylene carbons), 34.83 ( $\text{CH}_2\text{CHBr}$ ), 46.56 ( $\text{CHBr}$ ), 69.35 ( $\text{OCH}(\text{CH}_3)_2$ ), 169.32 ( $\text{COO}$ ); MS (70 eV)  $m/z$  (%) = 349 (2) [ $\text{M}^+$ ], 306 (25) [ $\text{M}^+$  - isopropyl], 269 (21) [ $\text{M}^+$  - Br], 227 (65) [ $\text{M}^+$  - Br -  $\text{C}_3\text{H}_6$ ], 57 (100) [ $\text{C}_4\text{H}_9^+$ ], 55 (89) [ $\text{C}_4\text{H}_7^+$ ].

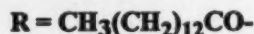
4.5.2. General procedure for 5'-*O*-esterification of thymidine (8)

General Structure

A solution of the respective fatty acid (66d, 67b, 67c, 68c, 70, 1.03 mmol) and diethyl azodicarboxylate (DEAD, 164  $\mu\text{L}$ , 1.04 mmol) in dioxane (1.72 mL) was added dropwise during 90 min to a solution of thymidine (250 mg, 1.03 mmol) and triphenylphosphine (271 mg, 1.03 mmol) in anhydrous dioxane (1.72 mL) with stirring at 60  $^\circ\text{C}$ . The reaction was allowed to proceed with stirring for 3 h at 60  $^\circ\text{C}$ , and then the solvent was removed *in vacuo*. The residue obtained was purified by silica gel column chromatography and using chloroform and then chloroform/methanol (95:5, v/v) as eluent. Removal of the

solvent *in vacuo* afforded the respective ester derivative (81a-e) as a solid that was crystallized from ethyl acetate. The spectral and microanalytical data for 81a-e are listed below.

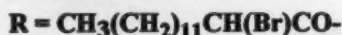
#### 4.5.2.1. 5'-O-(Myristoyl)thymidine (81a)



Yield: 222 mg, 48%; mp 147-149 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  0.86 (t,  $J = 6.8$  Hz, 3H,  $\text{CH}_2\text{CH}_3$ ), 1.14-1.42 (br m, 20H, methylene envelope), 1.48-1.74 (m, 2H,  $\text{CH}_2\text{CH}_2\text{COO}$ ), 1.92 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.25 (ddd,  $J_{2', 2''} = 13.5$ ,  $J_{2', 1'} = 6.5$  Hz,  $J_{2', 3'} = 6.7$  Hz, 1H, H-2'), 2.34 (t,  $J = 7.4$  Hz, 2H,  $\text{CH}_2\text{COO}$ ), 2.38 (ddd,  $J_{2'', 2'} = 13.5$ ,  $J_{2'', 1'} = 6.5$ ,  $J_{2'', 3'} = 4.0$  Hz, 1H, H-2''), 3.47 (br s, 1H, 3'-OH), 4.15 (ddd,  $J_{4', 5'} = 3.5$ ,  $J_{4', 5''} = 4.5$ ,  $J_{4', 3'} = 3.9$  Hz, 1H, H-4'), 4.25 (dd,  $J_{5', 5''} = 12.0$ ,  $J_{5', 4'} = 3.5$  Hz, 1H, H-5'), 4.40 (dd,  $J_{5'', 5'} = 12.0$  Hz,  $J_{5'', 4'} = 4.5$  Hz, 1H, H-5''), 4.32-4.40 (m, 1H, H-3'), 6.25 (t,  $J = 6.5$  Hz, 1H, H-1'), 7.26 (d,  $J_{6, 5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.10 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  12.06 (5- $\text{CH}_3$ ), 13.88 ( $\text{CH}_2\text{CH}_3$ ), 22.03, 24.34, 28.32, 28.64, 28.78, 28.93, 31.23, 33.32 [ $(\text{CH}_2)_{12}\text{CO}$ ], 40.34 (C-2'), 63.67 (C-5'), 70.22 (C-3'), 83.60 and 83.78 (C-4', C-1'), 109.60 (C-5), 135.76 (C-6), 150.35 (C-2 C=O), 163.59 (C-4 C=O), 172.72 (COO).

Anal. Calcd. for  $\text{C}_{24}\text{H}_{40}\text{N}_2\text{O}_6$ : C 63.69, H 8.90, N 6.19; found: C 63.68, H 8.94, N 6.14.

#### 4.5.2.2. ( $\pm$ )-5'-O-(2-Bromomyristoyl)thymidine (81b)



Yield: 88 mg, 16%; mp 113-115 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  0.86 (t,  $J = 6.25$  Hz, 3H,  $\text{CH}_2\text{CH}_3$ ), 1.20-1.42 (br m, 20H, methylene envelope), 1.94 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 1.92-2.12 (m, 2H,  $\text{CH}_2\text{-CHBr}$ ), 2.18 (br s, 1H, 3'-OH), 2.12-2.26 (m, 1H, H-2'), 2.38-2.49 (m, 1H, H-2''), 4.18-4.28 (m, 1H, H-4'), 4.25 (t,  $J = 7.2$  Hz, 1H, CHBr), 4.37

(dd,  $J_{5',5''} = 12.5$ ,  $J_{5',4'} = 3.2$  Hz, 1H, H-5'), 4.42-4.50 (m, 1H, H-3'), 4.56 (dd,  $J_{5',5''} = 12.5$ ,  $J_{5',4'} = 3.6$  Hz, 1H, H-5''), 6.28-6.36 (m, 1H, H-1'), 7.34 (d,  $J_{6,5-CH_3} = 1.3$  Hz, 1H, H-6), 9.60 (s, 1H, NH);  $^{13}\text{C}$  NMR (DMSO- $d_6$ )  $\delta$  12.09 (5- $\text{CH}_3$ ), 13.88 ( $\text{CH}_2\text{CH}_3$ ), 22.02, 26.41, 26.58, 28.12, 28.64, 28.78, 28.93, 31.23, 34.27, 34.42, 35.53 [ $(\text{CH}_2)_{11}\text{CHBrCO}$ ], 40.34 (C-2'), 47.78 (CHBr), 65.26 (C-5'), 70.09 (C-3'), 83.32, 83.77 (C-4', C-1'), 109.69 (C-5), 135.74 (C-6), 150.35 (C-2 C=O), 163.58 (C-4 C=O), COO (was not detected).

*Anal.* Calcd. for  $\text{C}_{24}\text{H}_{39}\text{BrN}_2\text{O}_6$ : C 54.23, H 7.39, N 5.27; found C 54.05, H 7.53, N 5.16.

#### 4.5.2.3. 5'-O-(11-Bromoundecanoyl)thymidine (81c)



Yield: 154 mg, 30%; mp 121-123 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.20-1.52 (br m, 12H, methylene envelope), 1.56-1.74 (m, 2H,  $\text{CH}_2\text{CH}_2\text{COO}$ ), 1.80-1.90 (m, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 1.94 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.09-2.22 (m, 1H, H-2'), 2.36 (t,  $J = 6.25$  Hz, 2H,  $\text{CH}_2\text{COO}$ ), 2.28-2.42 (m, 1H, H-2''), 2.43 (d,  $J = 5.7$  Hz, 1H, 3'-OH), 3.41 (t,  $J = 6.1$  Hz, 2H,  $\text{CH}_2\text{Br}$ ), 4.15 (ddd,  $J_{4',5'} = 3.5$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',3'} = 3.9$  Hz, 1H, H-4'), 4.28 (dd,  $J_{5',5''} = 12.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H, H-5'), 4.34-4.42 (m, 1H, H-3'), 4.42 (dd,  $J_{5',5''} = 12.0$ ,  $J_{5',4'} = 4.5$  Hz, 1H, H-5''), 6.29 (t,  $J = 6.5$  Hz, 1H, H-1'), 7.29 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.54 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.60 (5- $\text{CH}_3$ ), 24.82, 28.09, 28.66, 29.07, 29.13, 32.76, 33.96 [ $(\text{CH}_2)_9\text{Br}$ ], 34.15 ( $\text{CH}_2\text{COO}$ ), 40.46 (C-2'), 63.64 (C-5'), 71.64 (C-3'), 84.40, 85.21 (C-4', C-1'), 111.18 (C-5), 135.11 (C-6), 150.31 (C-2 C=O), 163.63 (C-4 C=O), 173.51 (COO).

*Anal.* Calcd. for  $\text{C}_{21}\text{H}_{33}\text{BrN}_2\text{O}_6$ : C 51.54, H 6.80, N 5.72; found C 51.48, H 6.92, N 5.65.

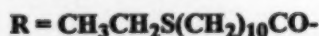
#### 4.5.2.4. 5'-O-(12-Bromododecanoyl)thymidine (81d)



5'-O-(12-Bromododecanoyl)thymidine (81d) was prepared by the general procedure in

32% yield (286 mg). A higher yield (55%) can be obtained using diisopropyl azodicarboxylate (DIAD) according to the following procedure. Thymidine (**8**, 1.00 g, 4.13 mmol) and triphenyl phosphine (1.62 g, 6.17 mmol) were dissolved in DMF (6 mL). A solution of DIAD (1.2 mL, 6.16 mmol) in DMF (3.0 mL) was added dropwise to the first solution with stirring followed by the dropwise addition of a solution of 12-bromododecanoic acid (**66d**, 1.15 g, 3.09 mmol) in DMF (3.0 mL). The reaction was allowed to proceed at 25 °C for 15 min with stirring and then an additional aliquot of  $\text{Ph}_3\text{P}$  (1.62 g, 6.17 mmol) and DIAD (1.2 mL, 6.16 mmol) was added. The reaction was allowed to proceed for 30 min at 25 °C with stirring, the solvent was removed *in vacuo* and the residue obtained was purified by silica gel column chromatography. Elution with chloroform-methanol (97:3, v/v) gave **5d** (1.1 g, 55%); mp 119-120;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.18-1.52 (br m, 14H, methylene envelope), 1.58-1.72 (m, 2H,  $\text{CH}_2\text{CH}_2\text{COO}$ ), 1.79-1.91 (m, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 1.93 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.04-2.20 (m, 1H, H-2'), 2.36 (t,  $J = 6.25$  Hz, 2H,  $\text{CH}_2\text{COO}$ ), 2.40-2.52 (m, 1H, H-2''), 3.19 (d,  $J = 5.8$  Hz, 1H, 3'-OH), 3.40 (t,  $J = 6.1$  Hz, 2H,  $\text{CH}_2\text{Br}$ ), 4.17 (ddd,  $J_{4',5'} = 3.5$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',3'} = 3.9$  Hz, 1H, H-4'), 4.28 (dd,  $J_{5',5''} = 12.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H, H-5'), 4.33-4.41 (m, 1H, H-3'), 4.40 (dd,  $J_{5'',5'} = 12.0$ ,  $J_{5'',4'} = 4.5$  Hz, 1H, H-5''), 6.30 (t,  $J = 6.8$  Hz, 1H, H-1'), 7.30 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.90 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.63 (5- $\text{CH}_3$ ), 24.83, 28.11, 28.69, 29.10, 29.17, 29.35, 32.78, 34.02 [ $(\text{CH}_2)_{10}\text{Br}$ ], 34.17 ( $\text{CH}_2\text{COO}$ ), 40.41 (C-2'), 63.68 (C-5'), 71.61 (C-3'), 84.43, 85.21 (C-4', C-1'), 111.21 (C-5), 135.13 (C-6), 150.39 (C-2 C=O), 163.69 (C-4 C=O), 173.54 (COO). *Anal.* Calcd. for  $\text{C}_{22}\text{H}_{35}\text{BrN}_2\text{O}_6$ : C 52.49, H 7.01, N 5.56; found C 52.59, H 6.97, N 5.48.

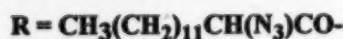
## 4.5.2.5. 5'-O-(11-Thioethylundecanoyl)thymidine (81e)



Yield: 127 mg, 26%; mp 125-127;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.24 (t,  $J = 7.4$  Hz, 3H,  $\text{CH}_2\text{CH}_3$ ), 1.50-1.80 (m, 4H,  $\text{CH}_2\text{CH}_2\text{COO}$ ,  $\text{SCH}_2\text{-CH}_2$ ), 1.20-1.50 (br m, 12H, methylene envelope), 1.94 (d,  $J_{5\text{-CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.08-2.22 (m, 1H, H-2'), 2.36 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{COO}$ ), 2.40-2.50 (m, 1H, H-2''), 2.51 (t,  $J = 7.5$  Hz, 2H,  $\text{SCH}_2\text{-CH}_2$ ), 2.52 (q,  $J = 7.4$  Hz, 2H,  $\text{SCH}_2\text{-CH}_3$ ), 2.69 (d,  $J = 5.0$  Hz, 1H, 3'-OH), 4.15 (ddd,  $J_{4', 5'} = 3.4$ ,  $J_{4', 5''} = 4.4$ ,  $J_{4', 3'} = 3.9$  Hz, 1H, H-4'), 4.27 (dd,  $J_{5', 5''} = 12.1$ ,  $J_{5', 4'} = 3.4$  Hz, 1H, H-5'), 4.33-4.42 (m, 1H, H-3'), 4.41 (dd,  $J_{5'', 5'} = 12.1$ ,  $J_{5'', 4'} = 4.4$  Hz, 1H, H-5''), 6.28 (t,  $J = 6.5$  Hz, 1H, H-1'), 7.28 (d,  $J_{6, 5\text{-CH}_3} = 1.3$  Hz, 1H, H-6), 8.78 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.63 (5- $\text{CH}_3$ ), 14.82 ( $\text{CH}_2\text{CH}_3$ ), 24.86, 25.96, 28.89, 29.11, 29.18, 29.32, 29.40, 29.63, 31.71, 34.19 (methylene carbons), 40.46 (C-2'), 63.58 (C-5'), 71.61 (C-3'), 84.37, 85.18 (C-4', C-1'), 111.14 (C-5), 135.08 (C-6), 150.14 (C-2 C=O), 163.42 (C-4 C=O), 173.51 (COO).

Anal. Calcd. for  $\text{C}_{23}\text{H}_{38}\text{N}_2\text{O}_6\text{S}$ : C 58.70, H 8.14, N 5.95 found C 58.32, H 7.97, N 5.91.

## 4.5.2.6. 5'-O-(2-Azidomyristoyl)thymidine (81f)



A solution of 5'-O-(2-bromomyristoyl)thymidine (81b, 50 mg, 0.094 mmol) and sodium azide (8.1 mg, 0.124 mmol) in dimethylformamide DMF (1 mL) was stirred at 25 °C for 8 h under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue obtained was purified by silica gel column chromatography. Elution with chloroform-methanol (98:2, v/v), removal of the solvent and recrystallization of the residue obtained from ethyl acetate afforded 5f (43 mg, 92%); mp 128-130 °C. 5'-O-(11-Azidoundecanoyl)thymidine (81g, 35.4 mg, 83.4%) and 5'-O-(12-azidododecanoyl)thymidine (81h, 40 mg, 91%) were synthesized using a procedure similar to that employed for the preparation of (81f) by using 5'-O-(11-



bromoundecanoyl)thymidine (81c) and 5'-O-(12-bromododecanoyl)thymidine (81d) as starting materials, respectively.  $^1\text{H}$  NMR ( $\text{CDCl}_3$  for 81f)  $\delta$  0.88 (t,  $J = 6.25$  Hz, 3H,  $\text{CH}_2\text{CH}_3$ ), 1.18-1.52 (br m, 20H, methylene envelope), 1.95 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 1.82-2.00 (m, 2H,  $\text{CH}_2\text{-CHN}_3$ ), 2.12-2.25 (m, 1H, H-2'), 2.42-2.52 (m, 1H, H-2''), 3.29 (br s, 1H, 3'-OH), 3.9 (dd,  $J = 4.0, J = 9.0$  Hz, 1H,  $\text{CHN}_3$ ), 4.18-4.24 (m, 1H, H-4'), 4.36-4.56 (br m, 3H, H-5', H-5'', H-3'), 6.26-6.36 (m, 1H, H-1'), 7.27 (d,  $J_{6, 5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 9.43 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.44 (5- $\text{CH}_3$ ), 14.07 ( $\text{CH}_2\text{CH}_3$ ), 22.64, 25.73, 28.99, 29.31, 29.46, 29.58, 31.35, 31.42, 31.88 (methylene carbons), 40.18 (C-2'), 62.26 ( $\text{CHN}_3$ ), 64.84 (C-5'), 71.62 (C-3'), 83.89, 85.20 (C-4', C-1'), 111.52 (C-5), 135.25 (C-6), 150.43 (C-2 C=O), 163.68 (C-4 C=O), 170.49 (COO); IR (KBr):  $\nu$  2110 ( $\text{N}_3$ )  $\text{cm}^{-1}$ .

*Anal.* Calcd. for  $\text{C}_{24}\text{H}_{39}\text{N}_5\text{O}_6$ : C 58.39, H 7.96, N 14.18; found C 57.96, H 7.77, N 13.76.

#### 4.5.2.7. 5'-O-(11-Azidoundecanoyl)thymidine (81g)



Yield: (35 mg, 83%); mp 98-99 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.50 (br m, 12H, methylene envelope), 1.54-1.80 (m, 4H,  $\text{CH}_2\text{CH}_2\text{COO}$ ,  $\text{CH}_2\text{CH}_2\text{N}_3$ ), 1.95 (d,  $J_{5-\text{CH}_3, 6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.09-2.22 (m, 1H, H-2'), 2.37 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{COO}$ ), 2.36-2.50 (m, 1H, H-2''), 2.63 (br, 1H, 3'-OH), 3.26 (t,  $J = 6.9$  Hz, 2H,  $\text{CH}_2\text{N}_3$ ), 4.16 (ddd,  $J_{4', 5'} = 3.5$ ,  $J_{4', 5''} = 4.5$ ,  $J_{4', 3'} = 3.9$  Hz, 1H, H-4'), 4.29 (dd,  $J_{5', 5''} = 12.0$ ,  $J_{5', 4'} = 3.5$  Hz, 1H, H-5'), 4.34-4.42 (m, 1H, H-3'), 4.42 (dd,  $J_{5'', 5'} = 12.0$ ,  $J_{5'', 4'} = 4.5$  Hz, 1H, H-5''), 6.29 (t,  $J = 6.5$  Hz, 1H, H-1'), 7.30 (d,  $J_{6, 5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.72 (s, 1H, NH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.62 (5- $\text{CH}_3$ ), 24.82, 26.65, 28.78, 29.06, 29.13, 29.25 (methylene carbons), 34.16 ( $\text{CH}_2\text{COO}$ ), 40.42 (C-2'), 51.45 ( $\text{CH}_2\text{N}_3$ ), 63.63 (C-5'),

71.61 (C-3'), 84.40, 85.20 (C-4', C-1'), 111.18 (C-5), 135.11 (C-6), 150.29 (C-2 C=O), 163.58 (C-4 C=O), 173.52 (COO); IR (KBr):  $\nu$  2090 (N<sub>3</sub>) cm<sup>-1</sup>.

*Anal.* Calcd. for C<sub>21</sub>H<sub>33</sub>N<sub>5</sub>O<sub>6</sub>: C 55.86, H 7.37, N 15.51; found C 55.84, H 7.34, N 15.18.

#### 4.5.2.8. 5'-O-(12-Azidododecanoyl)thymidine (81h)



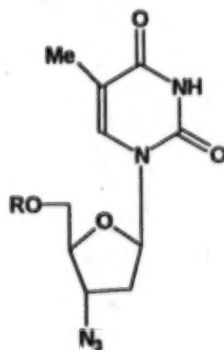
Yield: 40 mg, 91%; mp 100-102 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.10-1.36 (br m, 14H, methylene envelope), 1.40-1.74 (m, 4H, CH<sub>2</sub>CH<sub>2</sub>COO, CH<sub>2</sub>CH<sub>2</sub>N<sub>3</sub>), 1.87 (d,  $J_{5-CH_3, 6} = 1.3$  Hz, 3H, 5-CH<sub>3</sub>), 2.01-2.14 (m, 1H, H-2'), 2.24-2.42 (m, 1H, H-2''), 2.29 (t,  $J = 7.5$  Hz, 2H, CH<sub>2</sub>COO), 3.18 (t,  $J = 6.9$  Hz, 2H, CH<sub>2</sub>N<sub>3</sub>), 3.42 (br s, 1H, 3'-OH), 4.06 (ddd,  $J_{4',5'} = 3.5$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',3'} = 3.9$  Hz, 1H, H-4'), 4.20 (dd,  $J_{5',5''} = 12.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H, H-5'), 4.27-4.34 (m, 1H, H-3'), 4.34 (dd,  $J_{5'',5'} = 12.0$ ,  $J_{5'',4'} = 4.5$  Hz, 1H, H-5''), 6.20 (t,  $J = 6.5$  Hz, 1H, H-1'), 7.20 (d,  $J_{6,5-CH_3} = 1.3$  Hz, 1H, H-6), 8.14 (s, 1H, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  12.63 (5-CH<sub>3</sub>), 24.82, 26.67, 28.80, 29.08, 29.17, 29.37, 29.67 (methylene carbons), 34.17 (CH<sub>2</sub>COO), 40.41 (C-2'), 51.45 (CH<sub>2</sub>N<sub>3</sub>), 63.69 (C-5'), 71.61 (C-3'), 84.43, 85.21 (C-4', C-1'), 111.21 (C-5), 135.14 (C-6), 150.40 (C-2 C=O), 163.72 (C-4 C=O), 173.55 (COO); IR (KBr):  $\nu$  2110 (N<sub>3</sub>) cm<sup>-1</sup>.

*Anal.* Calcd. for C<sub>22</sub>H<sub>35</sub>N<sub>5</sub>O<sub>6</sub>: C 56.76, H 7.58, N 15.04; found C 57.06, H 7.45, N 14.86.

#### 4.5.3. General procedure used for 5'-O-esterification of AZT (6) and FLT (7)

A reaction mixture consisting of the appropriate fatty acid (1.3 mmol), oxalyl chloride (0.25 g, 1.95 mmol), and anhydrous benzene (18 mL) was stirred at room temperature (25 °C) for 1 h. The yellow solution thus obtained was evaporated to dryness under reduced pressure. The residual oil was dissolved in benzene (18 mL) and the solution was added dropwise to an ice-cold, stirred solution consisting of the AZT (0.34 g, 1.3 mmol) or the

FLT (0.32 g, 1.3 mmol), dimethylamino pyridine (DMAP) (0.23 g, 1.9 mmol) and anhydrous benzene (18 mL) under anhydrous conditions. The solution was stirred in an ice bath for 1 h and then refluxed in an oil bath for about 3 h. The mixture was cooled and diluted with benzene (72 mL). The organic solution was washed with saturated aqueous sodium carbonate ( $2 \times 11$  mL) and then with water ( $2 \times 11$  mL). The organic layer was dried over anhydrous sodium sulfate and was evaporated to dryness. The residue consisting of one major product was purified by silica gel chromatography using chloroform as eluent to yield the product. This procedure was used for the synthesis of most of ester analogs, unless noted otherwise.

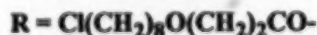


General Structure

#### 4.5.3.1. 5'-O-esters of AZT

##### 4.5.3.1.1. 4-Oxa derivatives

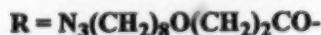
##### 4.5.3.1.1.1. 3'-Azido-2',3'-dideoxy-5'-O-(4-oxa-12-chlorododecanoyl)thymidine (82a)



Oil; yield (247 mg, 40%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.48 (br m, 8H, methylene envelope), 1.48-1.60 (m, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.70-1.86 (quintet,  $J = 7.3$ ,  $J = 6.9$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Cl}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.35 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2'',3'} = 7.6$ ,  $J_{2'',1'} = 6.2$  Hz, 1H, H-2''), 2.46 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2',3'} = 6.2$ ,  $J_{2',1'} = 6.2$  Hz, 1H, H-2'), 2.64 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.44 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.54 (t,  $J = 6.9$  Hz, 2H,  $\text{CH}_2\text{Cl}$ ), 3.73 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.08 (ddd,  $J_{4',3'} = 5.0$ ,  $J_{4',5''} = 3.7$ ,

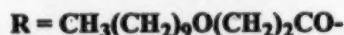
$J_{4',5'} = 3.0$  Hz, 1H, H-4'), 4.22 (ddd,  $J_{3',2''} = 7.6$ ,  $J_{3',2'} = 6.2$ ,  $J_{3',4'} = 5.0$  Hz, 1H, H-3'), 4.32 and 4.52 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 3.7$ ,  $J_{5',4'} = 3.0$  Hz, 1H each, H-5' and H-5''), 6.16 (t,  $J_{1',2''} = J_{1',2'} = 6.2$  Hz, 1H, H-1'), 7.27 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 8.58 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.53 (5- $\text{CH}_3$ ), 25.91, 26.77, 28.76, 29.20, 29.49, 32.53 (methylene carbons), 35.21 ( $\text{CH}_2\text{COO}$ ), 37.58 (C-2'), 45.08 ( $\text{CH}_2\text{Cl}$ ), 60.45 (C-3'), 63.11 (C-5'), 65.90 ( $\text{CH}_2\text{CH}_2\text{O}$ ), 71.31 ( $\text{OCH}_2\text{CH}_2\text{COO}$ ), 81.91 (C-4'), 85.20 (C-1'), 111.29 (C-5), 135.17 (C-6), 149.89 (C-2 C=O), 163.21 (C-4 C=O), 171.22 (COO).  
*Anal.* Calcd. for  $\text{C}_{21}\text{H}_{32}\text{ClN}_5\text{O}_6$ : C 51.90, H 6.64, N 14.41; found C 52.30, H 6.73, N 14.02.

4.5.3.1.1.2. 3'-Azido-2',3'-dideoxy-5'-O-(4-oxa-12-azidododecanoyl)thymidine (82b)



Oil; yield (132 mg, 41%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.46 (br m, 8H, methylene envelope), 1.46-1.72 (m, 4H,  $\text{CH}_2\text{CH}_2\text{N}_3$ ,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.36 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',3'} = 7.0$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.46 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2',3'} = 6.1$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 2.64 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.26 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{N}_3$ ), 3.43 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.73 (t,  $J = 6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.08 (ddd,  $J_{4',3'} = 5.0$ ,  $J_{4',5''} = 4.0$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.21 (ddd,  $J_{3',2''} = 7.0$ ,  $J_{3',2'} = 6.1$ ,  $J_{3',4'} = 5.0$  Hz, 1H, H-3'), 4.31 and 4.55 (two dd,  $J_{\text{gem}} = 12.5$ ,  $J_{5'',4'} = 4.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.16 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.27 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 8.45 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.56 (5- $\text{CH}_3$ ), 25.90, 28.77, 29.03, 29.23, 29.49, 29.67 (methylene carbons), 35.21 ( $\text{CH}_2\text{COO}$ ), 37.58 (C-2'), 51.44 ( $\text{CH}_2\text{N}_3$ ), 60.42 (C-3'), 63.11 (C-5'), 65.90 ( $\text{CH}_2\text{CH}_2\text{O}$ ), 71.31 ( $\text{OCH}_2\text{CH}_2\text{COO}$ ), 81.90 (C-4'), 85.18 (C-1'), 111.29 (C-5), 135.19 (C-6), 149.93 (C-2 C=O), 163.30 (C-4 C=O), 171.24 (COO).  
*Anal.* Calcd. for  $\text{C}_{21}\text{H}_{32}\text{N}_8\text{O}_6$ : C 51.21, H 6.55, N 22.75; found C 51.60, H 6.79, N 22.36.

## 4.5.3.1.1.3. 3'-Azido-2',3'-dideoxy-5'-O-(4-oxatetradecanoyl)thymidine (82c)

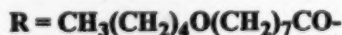


Oil; yield (342 mg, 56%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.20-1.40 (br m, 14H, methylene envelope), 1.46-1.62 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.35 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',3'} = 7.6$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.45 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2',1'} = 6.3$ ,  $J_{2',3'} = 5.4$  Hz, 1H, H-2'), 2.64 (t,  $J = 6.1$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 3.43 (t,  $J = 6.6$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.72 (t,  $J = 6.1$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.07 (ddd,  $J_{4',3'} = 5.2$ ,  $J_{4',5''} = 3.9$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.26 (ddd,  $J_{3',2''} = 7.6$ ,  $J_{3',2'} = 5.4$ ,  $J_{3',4'} = 5.2$  Hz, 1H, H-3'), 4.29 and 4.50 (two dd,  $J_{\text{gem}} = 12.4$ ,  $J_{5'',4'} = 3.9$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.17 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.27 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 9.32 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.51 (5- $\text{CH}_3$ ), 14.04 ( $\text{CH}_3$ ), 22.60, 25.98, 29.23, 29.38, 29.50, 31.83 (methylene carbons), 35.17 ( $\text{CH}_2\text{COO}$ ), 37.53 (C-2'), 60.38 (C-3'), 63.08 (C-5'), 65.84 ( $\text{CH}_2\text{CH}_2\text{O}$ ), 71.37 ( $\text{OCH}_2\text{CH}_2\text{COO}$ ), 81.85 (C-4'), 85.10 (C-1'), 111.33 (C-5), 135.17 (C-6), 150.18 (C-2 C=O), 163.69 (C-4 C=O), 171.24 (COO).

Anal. Calcd. for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_6$ : C 57.60, H 7.78, N 14.60; found C 57.80, H 7.99, N 14.27.

## 4.5.3.1.2. 9-Substituted analogs

## 4.5.3.1.2.1. 3'-Azido-2',3'-dideoxy-5'-O-(9-oxatetradecanoyl)thymidine (82d)



Oil; yield (270 mg, 44%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.90 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.22-1.46 (br m, 10H, methylene envelope), 1.50-1.77 (br m, 6H,  $\text{CH}_2\text{CH}_2\text{O}$ ,  $\text{CH}_2\text{CH}_2\text{O}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.95 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.38 (t,  $J = 6.6$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.30-2.44 (ddd,  $J_{\text{gem}} = 13.7$ ,  $J_{2'',3'} = 7.2$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.49 (ddd,  $J_{\text{gem}} = 13.7$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.39 (dt,  $J = 6.5$  Hz, 4H,



$\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$ ), 4.09 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.20 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.31 and 4.39 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.13 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.23 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 9.48 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.57 (5- $\text{CH}_3$ ), 13.98 ( $\text{CH}_3$ ), 22.48, 24.72, 25.96, 28.30, 29.01, 29.38, 29.63 (methylene carbons), 34.02 ( $\text{CH}_2\text{COO}$ ), 37.56 (C-2'), 60.64 (C-3'), 63.16 (C-5'), 70.72 ( $\text{CH}_2\text{O}$ ), 70.92 ( $\text{CH}_2\text{O}$ ), 81.79 (C-4'), 85.50 (C-1'), 111.24 (C-5), 135.17 (C-6), 150.14 (C-2 C=O), 163.68 (C-4 C=O), 173.04 (COO).

*Anal.* Calcd. for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_6$ : C 57.60, H 7.78, N 14.60; found C 57.66, H 7.83, N 14.70.

#### 4.5.3.1.2.2. 3'-Azido-2',3'-dideoxy-5'-O-(9-thiatetradecanoyl)thymidine (82e)



Oil; yield (225 mg, 36%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (t,  $J = 7.1$  Hz, 3H,  $\text{CH}_3$ ), 1.22-1.44 (br m, 10H, methylene envelope), 1.50-1.72 (m, 6H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.92 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.36 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.28-2.40 (m, 1H, H-2'), 2.48 (dt,  $J = 7.5$  Hz, 4H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2$ ), 2.41-2.58 (m, 1H, H-2'), 4.06 (ddd,  $J_{4',3'} = 4.4$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.18 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.3$ ,  $J_{3',4'} = 4.4$  Hz, 1H, H-3'), 4.29 and 4.37 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.10 (t,  $J_{1',2''} = J_{1',2'} = 6.2$  Hz, 1H, H-1'), 7.23 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 9.46 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.55 (5- $\text{CH}_3$ ), 13.89 ( $\text{CH}_3$ ), 22.22, 24.67, 28.57, 28.76, 28.90, 29.32, 29.49, 31.03, 32.02, 32.10 (methylene carbons), 33.98 ( $\text{CH}_2\text{COO}$ ), 37.53 (C-2'), 60.62 (C-3'), 63.14 (C-5'), 81.74 (C-4'), 85.48 (C-1'), 111.21 (C-5), 135.17 (C-6), 150.11 (C-2 C=O), 163.66 (C-4 C=O), 172.98 (COO).

*Anal.* Calcd. for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_5\text{S}$ : C 55.74, H 7.52, N 14.13; found C 55.68, H 7.64, N 13.76.

#### 4.5.3.1.3. 12-Substituted analogs

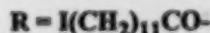
##### 4.5.3.1.3.1. 3'-Azido-2',3'-dideoxy-5'-O-(12-fluorododecanoyl)thymidine (83a)



Oil, yield (317 mg, 53%);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.16-1.50 (br m, 14H, methylene envelope), 1.56-1.80 (m, 4H,  $CH_2CH_2CO$ ,  $CH_2CH_2F$ ), 1.94 (d,  $J_{5-CH_3,6} = 1.3$  Hz, 3H, 5- $CH_3$ ), 2.36 (t,  $J = 7.3$  Hz, 2H,  $CH_2CO$ ), 2.26-2.42 (ddd,  $J_{gem} = 13.8$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{gem} = 13.8$ ,  $J_{2',3'} = 6.4$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 4.07 (ddd,  $J_{4',3'} = 5.0$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',5'} = 3.7$  Hz, 1H, H-4'), 4.18 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.4$ ,  $J_{3',4'} = 5.0$  Hz, 1H, H-3'), 4.30 and 4.38 (two dd,  $J_{gem} = 12.2$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.7$  Hz, 1H each, H-5' and H-5''), 4.44 (t,  $J_{gem} = 47.0$ ,  $J_{vic} = 6.1$  Hz, 2H,  $CH_2F$ ), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-CH_3} = 1.3$  Hz, 1H, H-6), 8.61 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.57 (5- $CH_3$ ), 24.79, 25.05, 25.12, 29.05, 29.14, 29.32, 29.37, 29.64, 30.21, 30.47 (methylene carbons), 34.06 ( $CH_2COO$ ), 37.59 (C-2'), 60.64 (C-3'), 63.14 (C-5'), 81.79 (C-4'), 84.17 ( $J_{C,F} = 164.6$  Hz, C-12), 85.47 (C-1'), 111.24 (C-5), 135.14 (C-6), 150.09 (C-2 C=O), 163.62 (C-4 C=O), 173.09 (COO);  $^{19}F$  NMR ( $CDCl_3$ )  $\delta$  -56.78 (dddd,  $J_{gem} = 47.0$ ,  $J_{vic} = 24.9$  Hz, 1F,  $CH_2F$ ).

Anal. Calcd. for  $C_{22}H_{34}FN_5O_5$ : C 56.52, H 7.32, N 14.98; found C 56.92, H 7.71, N 14.59.

##### 4.5.3.1.3.2. 3'-Azido-2',3'-dideoxy-5'-O-(12-iodododecanoyl)thymidine (83b)



**Method a:** General procedure, Yellow oil; yield (350 mg, 48%).

**Method b:** A mixture of 3'-azido-2',3'-deoxy-5'-O-(12-bromododecanoyl)thymidine (83d, 87.7 mg, 0.17 mmol) and sodium iodide (178 mg, 1.19 mmol) in dry acetone (1.18 mL) was stirred for 24 h in room temperature. After filtration the solvent was evaporated to dryness. The residue was dissolved in water (60 mL) and extracted with chloroform. After drying with sodium sulfate and evaporation of chloroform, the residue consisting of one major product was purified by chromatography over silica gel using chloroform as eluent

to yield 69 mg (83b, 72%) of product as yellow oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.30-1.60 (br m, 14H, methylene envelope), 1.64-1.80 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.87 (quint,  $J = 7.1$  Hz,  $J = 6.7$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{I}$ ), 1.99 (d,  $J_{5-\text{CH}_3,6} = 1.2$  Hz, 3H, 5- $\text{CH}_3$ ), 2.47 (t,  $J = 7.1$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.34-2.50 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2'',3'} = 7.1$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.57 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.29 (t,  $J = 7.1$  Hz, 2H,  $\text{CH}_2\text{I}$ ), 4.20 (ddd,  $J_{4',3'} = 4.6$ ,  $J_{4',5''} = 4.6$ ,  $J_{4',5'} = 3.7$  Hz, 1H, H-4'), 4.28 (ddd,  $J_{3',2''} = 7.1$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.6$  Hz, 1H, H-3'), 4.41 and 4.49 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.6$ ,  $J_{5',4'} = 3.7$  Hz, 1H each, H-5' and H-5''), 6.23 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.34 (d,  $J_{6,5-\text{CH}_3} = 1.2$  Hz, 1H, H-6), 8.90 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.14 ( $\text{CH}_2\text{I}$ ), 12.63 (5- $\text{CH}_3$ ), 24.85, 28.54, 28.90, 29.17, 29.25, 29.46, 30.53, 33.61, (methylene carbons), 34.17 ( $\text{CH}_2\text{COO}$ ), 37.70 (C-2'), 45.14 ( $\text{CH}_2\text{Cl}$ ), 60.78 (C-3'), 63.18 (C-5'), 81.91 (C-4'), 85.56 (C-1'), 111.22 (C-5), 135.11 (C-6), 149.81 (C-2 C=O), 163.21 (C-4 C=O), 172.98 (COO).

*Anal.* Calcd. for  $\text{C}_{22}\text{H}_{34}\text{IN}_5\text{O}_5$ : C 45.92, H 5.96, N 12.17, found C 46.30, H 5.99, N 12.17.

#### 4.5.3.1.3.3. 3'-Azido-2',3'-dideoxy-5'-O-(12-chlorododecanoyl)thymidine (83c)

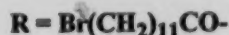


Oil; yield (399 mg, 65%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.16-1.50 (br m, 14H, methylene envelope), 1.56-1.70 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.76 (quintet,  $J = 7.8$ ,  $J = 7.0$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Cl}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.4$  Hz, 3H, 5- $\text{CH}_3$ ), 2.37 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.28-2.40 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2'',3'} = 7.1$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.52 (t,  $J = 7$  Hz, 2H,  $\text{CH}_2\text{Cl}$ ), 4.07 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.1$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.30 and 4.38 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.13 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-\text{CH}_3} = 1.4$  Hz, 1H, H-6), 9.54 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.54 (5- $\text{CH}_3$ ), 24.74,

26.77, 28.75, 29.01, 29.10, 29.31, 32.55 (methylene carbons), 34.02 (CH<sub>2</sub>COO), 37.55 (C-2'), 45.10 (CH<sub>2</sub>Cl), 60.61 (C-3'), 63.13 (C-5'), 81.76 (C-4'), 85.44 (C-1'), 111.21 (C-5), 135.14 (C-6), 150.15 (C-2 C=O), 163.73 (C-4 C=O), 173.06 (COO).

*Anal.* Calcd. for C<sub>22</sub>H<sub>34</sub>ClN<sub>5</sub>O<sub>5</sub>: C 54.60, H 7.08, N 14.47; found C 54.66, H 7.08, N 14.47.

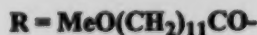
#### 4.5.3.1.3.4. 3'-Azido-2',3'-dideoxy-5'-O-(12-bromododecanoyl)thymidine (83d)



Oil; yield (399 mg, 59%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.22-1.54 (br m, 14H, methylene envelope), 1.60-1.74 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CO), 1.87 (quintet, *J* = 7.7, *J* = 6.8 Hz, 2H, CH<sub>2</sub>CH<sub>2</sub>Br), 1.96 (d, *J*<sub>5-CH<sub>3</sub>,6</sub> = 1.3 Hz, 3H, 5-CH<sub>3</sub>), 2.39 (t, *J* = 7.3 Hz, 2H, CH<sub>2</sub>CO), 2.30-2.44 (ddd, *J*<sub>gem</sub> = 13.9, *J*<sub>2'',3'</sub> = 7.5, *J*<sub>2'',1'</sub> = 6.3 Hz, 1H, H-2''), 2.38 (ddd, *J*<sub>gem</sub> = 13.9, *J*<sub>2',3'</sub> = 6.5, *J*<sub>2',1'</sub> = 6.3 Hz, 1H, H-2'), 3.42 (t, *J* = 6.8 Hz, 2H, CH<sub>2</sub>Br), 4.10 (ddd, *J*<sub>4',3'</sub> = 4.4, *J*<sub>4',5''</sub> = 4.2, *J*<sub>4',5'</sub> = 3.5 Hz, 1H, H-4'), 4.21 (ddd, *J*<sub>3',2''</sub> = 7.5, *J*<sub>3',2'</sub> = 6.5, *J*<sub>3',4'</sub> = 4.4 Hz, 1H, H-3'), 4.32 and 4.40 (two dd, *J*<sub>gem</sub> = 12.2, *J*<sub>5'',4'</sub> = 4.2, *J*<sub>5',4'</sub> = 3.5 Hz, 1H each, H-5' and H-5''), 6.14 (t, *J*<sub>1',2''</sub> = *J*<sub>1',2'</sub> = 6.3 Hz, 1H, H-1'), 7.24 (d, *J*<sub>6,5-CH<sub>3</sub></sub> = 1.3 Hz, 1H, H-6), 9.60 (s, 1H, N-H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.56 (5-CH<sub>3</sub>), 24.75, 26.77, 28.06, 28.63, 29.02, 29.11, 29.29, 32.55, 32.73 (methylene carbons), 34.02 (CH<sub>2</sub>COO), 37.56 (C-2'), 45.10 (CH<sub>2</sub>Br), 60.61 (C-3'), 63.13 (C-5'), 81.76 (C-4'), 85.44 (C-1'), 111.21 (C-5), 135.14 (C-6), 159.17 (C-2 C=O), 163.74 (C-4 C=O), 173.06 (COO).

*Anal.* Calcd. for C<sub>22</sub>H<sub>34</sub>BrN<sub>5</sub>O<sub>5</sub>: C 50.00, H 6.49, N 13.25; found C 50.29, H 6.52, N 12.86.

#### 4.5.3.1.3.5. 3'-Azido-2',3'-dideoxy-5'-O-(12-methoxydodecanoyl)thymidine (83e)

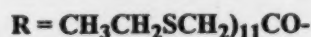


Oil; yield (342 mg, 56%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.22-1.48 (br m, 14H, methylene

envelope), 1.50-1.76 (m, 4H,  $\text{CH}_2\text{CH}_2\text{O}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.4$  Hz, 3H, 5- $\text{CH}_3$ ), 2.39 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.32-2.44 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2'',3'} = 7.2$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.50 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2',3'} = 6.6$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.35 (s, 3H,  $\text{OCH}_3$ ), 3.38 (t,  $J = 6.6$  Hz, 2H,  $\text{OCH}_2$ ), 4.10 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.3$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.23 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.6$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.32 and 4.40 (two dd,  $J_{\text{gem}} = 12.3$ ,  $J_{5'',4'} = 4.3$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.15 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.24 (d,  $J_{6,5-\text{CH}_3} = 1.4$  Hz, 1H, H-6), 9.94 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.48 (5- $\text{CH}_3$ ), 24.70, 25.98, 28.98, 29.07, 29.26, 29.32, 29.46 (methylene carbons), 33.98 ( $\text{CH}_2\text{COO}$ ), 37.48 (C-2'), 58.31 ( $\text{OCH}_3$ ), 60.59 (C-3'), 63.10 (C-5'), 72.81 ( $\text{OCH}_2$ ), 81.71 (C-4'), 85.39 (C-1'), 111.15 (C-5), 135.10 (C-6), 150.23 (C-2 C=O), 163.81 (C-4 C=O), 173.01 ( $\text{COO}$ ).

*Anal.* Calcd. for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_6$ : C 57.60, H 7.78, N 14.60; found C 57.65, H 7.83, N 14.31.

#### 4.5.3.1.3.6. 3'-Azido-2',3'-dideoxy-5'-O-(12-thioethyldodecanoyl)thymidine (83f)



Oil; yield (403 mg, 62%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.26 (t,  $J = 7.1$  Hz, 3H,  $\text{CH}_3$ ), 1.20-1.48 (br m, 14H, methylene envelope), 1.50-1.76 (m, 4H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.95 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.38 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.28-2.41 (m, 1H, H-2''), 2.51 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{S}$ ), 2.53 (q,  $J = 7.1$  Hz, 2H,  $\text{CH}_3\text{CH}_2\text{S}$ ), 2.44-2.64 (m, 1H, H-2'), 4.09 (ddd,  $J_{4',3'} = 4.4$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.3$ ,  $J_{3',4'} = 4.4$  Hz, 1H, H-3'), 4.31 and 4.39 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.13 (t,  $J_{1',2''} = J_{1',2'} = 6.2$  Hz, 1H, H-1'), 7.23 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.70 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.51 (5- $\text{CH}_3$ ), 14.70 ( $\text{CH}_3$ ), 24.70, 25.78, 28.80, 28.99, 29.08, 29.26, 29.34, 29.52 (methylene carbons), 31.54 ( $\text{CH}_2\text{S}$ ), 33.99 ( $\text{CH}_2\text{COO}$ ), 37.49 (C-2'), 60.57 (C-3'), 63.10 (C-5'), 81.70 (C-4'), 85.39 (C-1'), 111.16 (C-5), 135.12 (C-6), 150.20 (C-2 C=O),



163.80 (C-4 C=O), 173.01 (COO).

*Anal.* Calcd. for  $C_{24}H_{39}N_5O_5S$ : C 56.56, H 7.71, N 13.74; found C 56.69, H 7.86, N 13.49.

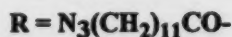
**4.5.3.1.3.7. 3'-Azido-2',3'-dideoxy-5'-O-(12-phenyldodecanoyl)thymidine (83g)**



Oil; yield (368 mg, 55 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.50 (br m, 14H, methylene envelope), 1.52-1.76 (m, 4H,  $\text{CH}_2\text{CH}_2\text{Ph}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.39 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.30-2.44 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2'',3'} = 7.2$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.50 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2',3'} = 6.2$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 2.62 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{Ph}$ ), 4.10 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.21 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.2$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.32 and 4.40 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.14 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 7.14-7.22 (m, 2H, aromatic), 7.23-7.38 (m, 3H, aromatic), 9.18 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.57 (5- $\text{CH}_3$ ), 24.80, 27.29, 29.08, 29.16, 29.26, 29.37, 29.44, 29.49, 31.44 (methylene carbons), 34.08 ( $\text{CH}_2\text{COO}$ ), 35.93 ( $\text{CH}_2\text{Ph}$ ), 37.61 (C-2'), 60.65 (C-3'), 63.13 (C-5'), 81.81 (C-4'), 85.46 (C-1'), 111.26 (C-5), 125.50, 128.16, 128.35 (aromatic carbons), 135.11 (C-6), 142.90 (aromatic quaternary), 150.03 (C-2 C=O), 163.51 (C-4 C=O), 173.09 (COO).

*Anal.* Calcd. for  $C_{28}H_{39}N_5O_5$ : C 63.10, H 7.48, N 13.32; found C 63.50, H 7.47, N 13.01.

**4.5.3.1.3.8. 3'-Azido-2',3'-dideoxy-5'-O-(12-azidododecanoyl)thymidine (83h)**



3'-Azido-5'-O-(12-iodomyristoyl)thymidine (83b, 32.9 mg, 0.057 mmol) and sodium azide (4.9 mg, 0.076 mmol) in DMF (0.58 mL) were stirred for 18 h. under nitrogen. The solvent was removed *in vacuo* and the residue was dissolved in water (2 mL) and

extracted with chloroform (3 × 2 mL). After drying with sodium sulfate and evaporation of chloroform, the residue consisting of one major product was purified by chromatography over silica gel using chloroform as eluent to yield 22 mg (80%) of product (**83h**) as a colorless foam.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.14-1.50 (br m, 14H, methylene envelope), 1.50-1.78 (m, 4H,  $\text{CH}_2\text{CH}_2\text{N}_3$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.38 (t,  $J = 7.2$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.26-2.42 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',3'} = 7.0$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2',3'} = 6.1$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.26 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{N}_3$ ), 4.08 (ddd,  $J_{4',3'} = 5.0$ ,  $J_{4',5''} = 4.0$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.0$ ,  $J_{3',2'} = 6.1$ ,  $J_{3',4'} = 4.0$  Hz, 1H, H-3'), 4.30 and 4.39 (two dd,  $J_{\text{gem}} = 12.5$ ,  $J_{5'',4'} = 4.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.13 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.23 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 8.52 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.60 (5- $\text{CH}_3$ ), 24.81, 26.67, 28.80, 29.08, 29.16, 29.38 (methylene carbons), 34.08 ( $\text{CH}_2\text{COO}$ ), 37.62 (C-2'), 51.47 ( $\text{CH}_2\text{N}_3$ ), 60.65 (C-3'), 63.13 (C-5'), 81.82 (C-4'), 85.45 (C-1'), 111.24 (C-5), 135.10 (C-6), 149.82 (C-2 C=O), 163.18 (C-4 C=O), 173.08 (COO).  
*Anal.* Calcd. for  $\text{C}_{22}\text{H}_{34}\text{N}_8\text{O}_5$ : C 53.87, H 6.99, N 22.84; found C 54.23, H 7.12, N 22.44.

#### 4.5.3.1.3.9. 3'-Azido-2',3'-dideoxy-5'-O-(12-phenoxydodecanoyl)thymidine (**83i**)

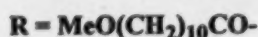
$\text{R} = \text{PhO}(\text{CH}_2)_{11}\text{CO}-$

Oil, yield (307 mg, 46 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.52 (br m, 14H, methylene envelope), 1.58-1.72 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.78 (quintet,  $J = 7.8$ ,  $J = 6.5$  Hz, 2H  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.2$  Hz, 3H, 5- $\text{CH}_3$ ), 2.37 (t,  $J = 7.8$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.28-2.42 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2'',3'} = 7.4$ ,  $J_{2'',1'} = 6.4$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2',3'} = 6.2$ ,  $J_{2',1'} = 6.4$  Hz, 1H, H-2'), 3.96 (t,  $J = 6.5$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.08 (ddd,  $J_{4',5''} = 4.5$ ,  $J_{4',3'} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.4$ ,  $J_{3',2'} = 6.2$ ,  $J_{3',4'} = 4.2$  Hz, 1H, H-3'), 4.30 and 4.38 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.5$

Hz, 1H each, H-5' and H-5''), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.4$  Hz, 1H, H-1'), 6.82-7.00 (m, 2H, aromatic), 7.22 (d,  $J_{6,5-CH_3} = 1.2$  Hz, 1H, H-6), 7.18-7.36 (m, 3H, aromatic), 9.30 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.59 (5- $CH_3$ ), 24.79, 29.01, 26.08, 29.08, 29.16, 29.25, 29.33, 29.45 (methylene carbons), 34.08 ( $CH_2COO$ ), 37.60 (C-2'), 60.64 (C-3'), 63.14 (C-5'), 67.84 ( $CH_2O$ ), 81.80 (C-4'), 85.47 (C-1'), 111.26 (C-5), 114.49, 120.41 (*meta* and *para* aromatic carbons), 129.36 (*ortho* aromatic carbons), 135.14 (C-6), 150.09 (C-2 C=O), 159.10 (quaternary aromatic), 163.60 (C-4 C=O), 173.09 (COO).  
*Anal.* Calcd. for  $C_{28}H_{39}N_5O_6$ : C 62.09, H 7.26, N 12.93; found C 61.79, H 7.28, N 12.56.

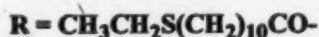
#### 4.5.3.1.4. 11-Substituted analogs

##### 4.5.3.1.4.1. 3'-Azido-2',3'-dideoxy-5'-O-(11-methoxyundecanoyl)thymidine (84a)



Oil; yield (361 mg, 61%);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.22-1.42 (br m, 12H, methylene envelope), 1.50-1.73 (m, 4H,  $CH_2CH_2O$ ,  $CH_2CH_2CO$ ), 1.94 (d,  $J_{5-CH_3,6} = 1.4$  Hz, 3H, 5- $CH_3$ ), 2.38 (t,  $J = 7.2$  Hz, 2H,  $CH_2CO$ ), 2.30-2.42 (ddd,  $J_{gem} = 13.8$ ,  $J_{2'',3'} = 7.1$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.49 (ddd,  $J_{gem} = 13.8$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.34 (s, 3H,  $OCH_3$ ), 3.38 (t,  $J = 6.6$  Hz, 2H,  $OCH_2$ ), 4.09 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.3$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.22 (ddd,  $J_{3',2''} = 7.1$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.31 and 4.39 (two dd,  $J_{gem} = 12.3$ ,  $J_{5'',4'} = 4.3$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.14 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.24 (d,  $J_{6,5-CH_3} = 1.4$  Hz, 1H, H-6), 9.88 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.51 (5- $CH_3$ ), 24.70, 25.97, 28.98, 29.07, 29.20, 29.30, 29.34, 29.46 (methylene carbons), 33.95 ( $CH_2COO$ ), 37.49 (C-2'), 58.34 ( $OCH_3$ ), 60.58 (C-3'), 63.10 (C-5'), 72.80 ( $OCH_2$ ), 81.70 (C-4'), 85.39 (C-1'), 111.17 (C-5), 135.11 (C-6), 150.24 (C-2 C=O), 163.84 (C-4 C=O), 173.03 (COO).  
*Anal.* Calcd. for  $C_{22}H_{35}N_5O_6$ : C 56.76, H 7.58, N 15.04; found C 56.77, H 7.75, N 14.81.

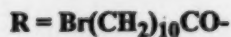
## 4.5.3.1.4.2. 3'-Azido-2',3'-dideoxy-5'-O-(11-thioethylundecanoyl)thymidine (84b)



Oil; yield (405 mg, 64 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.25 (t,  $J = 7.1$  Hz, 3H,  $\text{CH}_3$ ), 1.20-1.44 (br m, 12H, methylene envelope), 1.50-1.74 (m, 4H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.37 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.26-2.40 (m, 1H, H-2''), 2.51 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{S}$ ), 2.52 (q,  $J = 7.1$  Hz, 2H,  $\text{CH}_3\text{CH}_2\text{S}$ ), 2.42-2.60 (m, 1H, H-2'), 4.08 (ddd,  $J_{4',3'} = 4.4$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.18 (ddd,  $J_{3',2''} = 7.2$ ,  $J_{3',2'} = 6.3$ ,  $J_{3',4'} = 4.4$  Hz, 1H, H-3'), 4.29 and 4.38 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.2$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.48 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.63 (5- $\text{CH}_3$ ), 14.82 ( $\text{CH}_3$ ), 24.82, 25.93, 28.90, 29.11, 29.19, 29.34, 29.43, 29.62 (methylene carbons), 31.68 ( $\text{CH}_2\text{S}$ ), 34.11 ( $\text{CH}_2\text{COO}$ ), 37.64 (C-2'), 60.67 (C-3'), 63.16 (C-5'), 81.84 (C-4'), 85.45 (C-1'), 111.25 (C-5), 135.10 (C-6), 149.80 (C-2 C=O), 163.38 (C-4 C=O), 173.10 (COO).

Anal. Calcd. for  $\text{C}_{23}\text{H}_{37}\text{N}_5\text{O}_5\text{S}$ : C 55.74, H 7.52, N 14.13; found C 55.80, H 7.70, N 13.79.

## 4.5.3.1.4.3. 3'-Azido-2',3'-dideoxy-5'-O-(11-bromoundecanoyl)thymidine (84c)

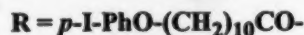


Oil; yield (290 mg, 44%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.50 (br m, 12H, methylene envelope), 1.60-1.72 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.86 (quintet,  $J = 7.7$ ,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 1.96 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.38 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.30-2.44 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.39 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.42 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{Br}$ ), 4.09 (ddd,  $J_{4',3'} = 4.4$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.20 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.4$  Hz, 1H, H-3'), 4.31 and 4.40 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$



Hz, 1H each, H-5' and H-5''), 6.13 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.23 (d,  $J_{6,5-CH_3} = 1.3$  Hz, 1H, H-6), 9.10 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.60 (5- $CH_3$ ), 24.77, 26.79, 28.77, 29.05, 29.11, 29.23, 32.57, 32.75 (methylene carbons), 34.05 ( $CH_2COO$ ), 37.61 (C-2'), 45.10 ( $CH_2Br$ ), 60.65 (C-3'), 63.16 (C-5'), 81.80 (C-4'), 85.48 (C-1'), 111.24 (C-5), 135.17 (C-6), 159.09 (C-2 C=O), 163.62 (C-4 C=O), 173.06 (COO).  
*Anal.* Calcd. for  $C_{21}H_{32}BrN_5O_5$ : C 49.03, H 6.27, N 13.61; found C 48.81, H 6.33, N 13.31.

4.5.3.1.4.4. 3'-Azido-2',3'-dideoxy-5'-O-(11-(4-iodophenoxy)undecanoyl)thymidine (84d)

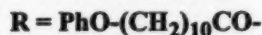


Oil; yield (530 mg, 62.4%);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.20-1.50 (br m, 12H, methylene envelope), 1.56-1.80 (m, 2H,  $CH_2CH_2CO$ ), 1.75 (quintet,  $J = 7.5$ ,  $J = 6.5$  Hz, 2H  $CH_2CH_2O$ ), 1.94 (d,  $J_{5-CH_3,6} = 1.1$  Hz, 3H, 5- $CH_3$ ), 2.37 (t,  $J = 7.4$  Hz, 2H,  $CH_2CO$ ), 2.26-2.42 (ddd,  $J_{gem} = 14.0$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{gem} = 14.0$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.91 (t,  $J = 6.5$  Hz, 2H,  $CH_2O$ ), 4.08 (ddd,  $J_{4',5'} = 4.5$ ,  $J_{4',3'} = 4.1$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.17 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.1$  Hz, 1H, H-3'), 4.30 and 4.38 (two dd,  $J_{gem} = 12.0$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 6.60-6.74 (m,  $J_{ortho} = 9.1$  Hz,  $J_{meta} = 2.8$  Hz, 2H, aromatic), 7.22 (d,  $J_{6,5-CH_3} = 1.1$  Hz, 1H, H-6), 7.48-7.60 (m,  $J_{ortho} = 9.1$  Hz,  $J_{meta} = 2.8$  Hz, 2H, aromatic), 9.16 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.60 (5- $CH_3$ ), 24.79, 25.91, 29.07, 29.15, 29.27, 29.40 (methylene carbons), 34.07 ( $CH_2COO$ ), 37.61 (C-2'), 60.64 (C-3'), 63.13 (C-5'), 68.07 ( $CH_2O$ ), 81.80 (C-4'), 82.36 (quaternary aromatic, I-C), 85.48 (C-1'), 111.24 (C-5), 116.93 (aromatic carbons), 135.14 (C-6), 138.11 (aromatic carbons), 150.03 (C-2 C=O), 158.98 (quaternary aromatic, C-O), 163.52 (C-4 C=O), 173.06 (COO).  
*Anal.* Calcd. for  $C_{27}H_{36}IN_5O_6$ : C 49.62, H 5.55, N 10.72; found C 49.77, H 5.54, N



10.30.

## 4.5.3.1.4.5. 3'-Azido-2',3'-dideoxy-5'-O-(11-phenoxyundecanoyl)thymidine (84e)



Oil, yield (307 mg, 46%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.52 (br m, 12H, methylene envelope), 1.56-1.70 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.76 (quintet,  $J = 7.5$ ,  $J = 6.8$  Hz, 2H  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.2$  Hz, 3H, 5- $\text{CH}_3$ ), 2.36 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.26-2.42 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2'',1'} = 6.4$  Hz, 1H, H-2''), 2.45 (ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2',3'} = 6.2$ ,  $J_{2',1'} = 6.4$  Hz, 1H, H-2'), 3.94 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.07 (ddd,  $J_{4',5''} = 4.5$ ,  $J_{4',3'} = 4.1$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.17 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.2$ ,  $J_{3',4'} = 4.1$  Hz, 1H, H-3'), 4.33 and 4.37 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.11 (t,  $J_{1',2''} = J_{1',2'} = 6.4$  Hz, 1H, H-1'), 6.82-7.00 (m, 2H, aromatic), 7.21 (d,  $J_{6,5-\text{CH}_3} = 1.2$  Hz, 1H, H-6), 7.18-7.34 (m, 3H, aromatic), 8.85 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.63 (5- $\text{CH}_3$ ), 24.82, 29.02, 29.10, 29.19, 29.32, 29.45 (methylene carbons), 34.11 ( $\text{CH}_2\text{COO}$ ), 37.64 (C-2'), 60.67 (C-3'), 63.16 (C-5'), 67.84 ( $\text{CH}_2\text{O}$ ), 81.83 (C-4'), 85.48 (C-1'), 111.27 (C-5), 114.50, 120.46 (*meta* and *para* aromatic carbons), 129.39 (*ortho* aromatic carbons), 135.14 (C-6), 149.97 (C-2 C=O), 159.13 (quaternary aromatic), 163.42 (C-4 C=O), 173.10 (COO).  
*Anal.* Calcd. for  $\text{C}_{27}\text{H}_{37}\text{N}_5\text{O}_6$ : C 61.46, H 7.07, N 13.27; found C 61.07, H 7.28, N 12.88.

## 4.5.3.1.4.6. 3'-Azido-2',3'-dideoxy-5'-O-(11-azidoundecanoyl)thymidine (84f)



Thymidine (8, 1.0 g, 4.1 mmol) and triphenylphosphine (1.6 g, 6.2 mmol) were dissolved in DMF (6.0 mL). To this stirred mixture a solution of diisopropyl azodicarboxylate (DIAD) (1.2 mL, 6.2 mmol) and 11-bromoundecanoic acid (67c, 109.6 mg, 4.1 mmol) in DMF (6.0 mL) are added drop by drop in 15 min and stirring is continued at room temperature for 15 min. The same quantity of triphenylphosphine in

DMF (1.0 mL) and DIAD in DMF (3.00 mL) was then added. After 30 min stirring at room temperature the solvent is removed under reduced pressure and the residue is applied to silica gel column chromatography. The product was purified using increasing concentrations of methanol in chloroform for elution. The product eluted at 2% methanol/chloroform. The solvent was removed under reduced pressure to yield 2,3'-anhydro-1-(5'-O-11-bromoundecanoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (**89**) 847 mg (44 %); as a yellow oil that was used directly in the next reaction without further purification.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.14-1.44 (br m, 12H, methylene envelope), 1.50-1.70 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.85 (quintet,  $J = 7.7$ ,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 1.97 (d,  $J_{5-\text{CH}_3,6} = 1.2$  Hz, 3H, 5- $\text{CH}_3$ ), 2.28 (t,  $J = 7.4$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.42-2.52 (ddd,  $J_{\text{gem}} = 13.0$ ,  $J_{2'',1'} = 4.1$ ,  $J_{2'',3'} = 2.9$  Hz, 1H, H-2''), 2.80 (dd,  $J_{\text{gem}} = 13.0$ ,  $J_{2',1'} = 4.1$  Hz 1H, H-2'), 3.40 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{Br}$ ), 4.21-4.37 (m, 2H, H-5' and H-5''), 4.37-4.50 (m, 1H, H-4'), 5.20 (br s, 1H, H-3'), 5.49 (d,  $J_{1',2''} = J_{1',2'} = 4.1$  Hz, 1H, H-1'), 6.96 (d,  $J_{6,5-\text{CH}_3} = 1.2$  Hz, 1H, H-6);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  13.16 (5- $\text{CH}_3$ ), 24.55, 27.93, 28.50, 28.84, 28.96, 29.08, 29.13, 32.61 (methylene carbons), 33.95 ( $\text{CH}_2\text{COO}$ ), 45.10 ( $\text{CH}_2\text{Br}$ ), 61.70 (C-5'), 76.79 (C-3'), 82.60 (C-4'), 87.55 (C-1'), 117.95 (C-5), 135.52 (C-6), 153.32 (C-4 C=O), 171.59 (C-2), 172.97 (COO).

2,3'-Anhydro-1-(5'-O-11-bromoundecanoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (**89**, 400 mg, 0.85 mmol) and sodium azide (139 mg, 2.1 mmol) in DMF (12 mL) were stirred at 60 °C under nitrogen for 12 h. The solvent was removed *in vacuo* and the residue was applied to silica gel column chromatography. The product was purified using an increasing concentration of methanol in chloroform for elution. The product eluted at 2% methanol/chloroform. The solvent was removed under reduced pressure to yield 2,3'-anhydro-1-(5'-O-11-azidoundecanoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (**90**, 298 mg, 81%); mp 111-113 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.44 (br m, 12H, methylene envelope), 1.50-1.70 (m, 4H,  $\text{CH}_2\text{CH}_2\text{CO}$ ,  $\text{CH}_2\text{CH}_2\text{N}_3$ ), 1.92 (d,  $J_{5-\text{CH}_3,6} = 1.2$  Hz, 3H, 5- $\text{CH}_3$ ), 2.26 (t,  $J = 7.6$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.42-2.54 (ddd,  $J_{\text{gem}} = 12.8$ ,  $J_{2'',1'} = 4.0$ ,  $J_{2'',3'} = 2.9$  Hz, 1H, H-2''), 2.80 (dd,  $J_{2',1'} = 4.1$ ,  $J_{\text{gem}} = 12.8$  Hz, 1H, H-2'), 3.26 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{N}_3$ ),

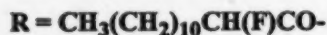
4.21-4.40 (m, 2H, H-5' and H-5''), 4.40-4.50 (m, 1H, H-4'), 5.22 (br s, 1H, H-3'), 5.54 (d,  $J_{1',2''} = J_{1',2'} = 4.0$  Hz, 1H, H-1'), 6.98 (d,  $J_{6,5-CH_3} = 1.2$  Hz, 1H, H-6);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  13.40 (5- $CH_3$ ), 24.81, 26.74, 28.088, 29.07, 29.11, 29.19, 29.23, 29.30, 29.39 (methylene carbons), 33.95 ( $CH_2COO$ ), 51.55 ( $CH_2N_3$ ), 61.74 (C-5'), 76.65 (C-3'), 82.87 (C-4'), 87.61 (C-1'), 118.68 (C-5), 134.95 (C-6), 153.22 (C-4 C=O), 171.61 (C-2), 172.99 (COO).

2,3'-Anhydro-1-(5'-O-11-azidoundecanoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (90, 297 mg, 0.068 mmol) and sodium azide (111 mg, 1.71 mmol) are suspended in DMF (8.9 mL) and the mixture was heated in the oil bath at 110 °C for 24 h under nitrogen. The residue was purified by silica gel column chromatography using 80:20 dichloromethane/ethylacetate as eluent to yield an oily product (84f, 292 mg, 87%);  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.20-1.44 (br m, 12H, methylene envelope), 1.50-1.74 (m, 4H,  $CH_2CH_2N_3$ ,  $CH_2CH_2CO$ ), 1.93 (d,  $J_{5-CH_3,6} = 1.1$  Hz, 3H, 5- $CH_3$ ), 2.36 (t,  $J = 7.2$  Hz, 2H,  $CH_2CO$ ), 2.26-2.42 (ddd,  $J_{gem} = 14.0$ ,  $J_{2'',3'} = 7.0$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{gem} = 14.0$ ,  $J_{2',3'} = 6.1$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 3.25 (t,  $J = 6.8$  Hz, 2H,  $CH_2N_3$ ), 4.07 (ddd,  $J_{4',3'} = 4.0$ ,  $J_{4',5''} = 4.0$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.0$ ,  $J_{3',2'} = 6.1$ ,  $J_{3',4'} = 4.0$  Hz, 1H, H-3'), 4.29 and 4.38 (two dd,  $J_{gem} = 12.5$ ,  $J_{5'',4'} = 4.0$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-CH_3} = 1.1$  Hz, 1H, H-6), 9.60 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.57 (5- $CH_3$ ), 24.76, 26.62, 28.75, 29.02, 29.10, 29.22, 29.30, 29.63 (methylene carbons), 34.04 ( $CH_2COO$ ), 37.58 (C-2'), 51.42 ( $CH_2N_3$ ), 60.62 (C-3'), 63.13 (C-5'), 81.79 (C-4'), 85.45 (C-1'), 111.23 (C-5), 135.14 (C-6), 150.03 (C-2 C=O), 163.54 (C-4 C=O), 173.06 (COO).

*Anal.* Calcd. for  $C_{21}H_{32}N_8O_5$ : C 52.93, H 6.77, N 23.51; found C 52.54, H 6.96, N 23.11.

#### 4.5.3.1.5. 2-Substituted analogs

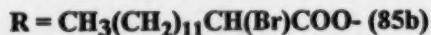
##### 4.5.3.1.5.1. (±)-3'-Azido-2',3'-dideoxy-5'-O-(2-fluorotetradecanoyl)thymidine (85a)



Oil; yield (352 mg, 56%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.87 (t,  $J = 6.2$  Hz, 3H,  $\text{CH}_3$ ), 1.16-1.54 (br m, 20H, methylene envelope), 1.82-2.06 (m, 2H,  $\text{CH}_2\text{CHF}$ ), 1.92 (d,  $J_{5-\text{CH}_3,6} = 1.0$  Hz, 3H, 5- $\text{CH}_3$ ), 2.32-2.52 (two ddd,  $J_{\text{gem}} = 13.5$ ,  $J_{2'',3'} = 6.6$ ,  $J_{2'',1'} = 6.6$  Hz,  $J_{2',1'} = 6.1$ ,  $J_{2',3'} = 4.5$  Hz, 2H, H-2'' and H-2'), 4.11 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 3.8$ ,  $J_{4',5'} = 2.9$  Hz, 1H, H-4'), 4.25 (ddd,  $J_{3',2''} = 6.6$ ,  $J_{3',2'} = 4.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.34-4.56 (ddd,  $J_{\text{gem}} = 12.0$ ,  $J_{5'',4'} = 3.8$ ,  $J_{5',4'} = 2.9$  Hz, 2H, H-5' and H-5''), 4.98 (two dddd,  $J_{\text{gem}} = 48.5$ ,  $J_{\alpha,\beta} = 7.2$ ,  $J_{\alpha,\beta'} = 4.5$ ,  $J_{\alpha,\gamma} = 2$  Hz, 1H, CHF), 6.19-6.26 (m,  $J_{1',2''} = 6.6$ ,  $J_{1',2'} = 6.1$  Hz, 1H, H-1'), 7.26 (d,  $J_{6,5-\text{CH}_3} = 1.0$  Hz, 1H, H-6), 9.60 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.27 (5- $\text{CH}_3$ ), 14.02 ( $\text{CH}_3$ ), 22.60, 24.38, 28.97, 29.26, 29.41, 29.55, 31.83, 32.31, 32.59 (methylene carbons), 37.27 (C-2'), 60.69 (C-3'), 64.13 (C-5'), 81.34 (C-4'), 85.09 (C-1'), 89.25 ( $J_{\text{C,F}} = 187.5$  Hz, CHF), 111.71 (C-5), 135.18 (C-6), 150.27 (C-2 C=O), 163.66 (C-4 C=O), 169.59 ( $J_{\text{C,F}} = 22.6$  Hz, COO).

Anal. Calcd. for  $\text{C}_{24}\text{H}_{38}\text{FN}_5\text{O}_5 \cdot \text{H}_2\text{O}$ : C 56.12, H 7.46, N 13.64; found C 56.13, H 7.59, N 13.40.

##### 4.5.3.1.5.2. (±)-3'-Azido-2',3'-dideoxy-5'-O-(2-bromotetradecanoyl)thymidine (85b)



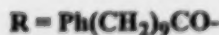
Yellow oil; using the general procedure; yield (19 mg, 3%); Because of the low yield in the general procedure, the following method was used: A mixture of 1,1'-carbonyldiimidazole (114 mg, 0.7 mmol) and methyl iodide (81  $\mu\text{L}$ , 1.3 mmol) in dry  $\text{CH}_3\text{CN}$  (15 mL) was stirred for 2 h at room temperature. 2-Bromomyristic acid (68c, 200 mg, 0.7 mmol) was added to the solution during 15 min (carbon dioxide gas was generated immediately) followed by AZT (174 mg, 0.7 mmol). The mixture was stirred at room temperature for 39 h and the solvent was evaporated to dryness. The residue consisting of one major product (85b) was purified by chromatography over silica gel

using first hexane and then chloroform as eluent to yield 159 mg (44%) of product as a yellow oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.84 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.14-1.50 (br m, 20H, methylene envelope), 1.90-2.08 (m, 2H,  $\text{CH}_2\text{CHBr}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.29-2.51 (two ddd,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2',3'} = 6.5$ ,  $J_{2'',1'} = J_{2',1'} = 6.3$  Hz, 2H, H-2'' and H-2'), 4.04-4.10 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.0$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.20-4.27 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.26 (t,  $J = 7.3$  Hz, 1H,  $\text{CHBr}$ ), 4.32-4.47 (ddd,  $J_{\text{gem}} = 12.0$ ,  $J_{5'',4'} = 4.0$ ,  $J_{5',4'} = 3.5$  Hz, 2H, H-5' and H-5''), 6.40-6.90 (m,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.24 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 9.30 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.56 (5- $\text{CH}_3$ ), 14.07 ( $\text{CH}_3$ ), 22.64, 27.22, 28.76, 29.29, 29.43, 29.58, 31.88, 34.73 (methylene carbons), 37.43 (C-2'), 45.56 ( $\text{CHBr}$ ) 60.37 (C-3'), 64.20 (C-5'), 81.54 (C-4'), 85.06 (C-1'), 111.50 (C-5), 135.11 (C-6), 149.90 (C-2 C=O), 163.24 (C-4 C=O), 169.31 (COO).

*Anal.* Calcd. for  $\text{C}_{24}\text{H}_{38}\text{BrN}_5\text{O}_5 \cdot 1.5 \text{H}_2\text{O}$ : C 49.40, H 7.08, N 12.00; found C 49.09, H 6.62, N 11.78.

#### 4.5.3.1.6. Other 5'-O-ester analogs of AZT

##### 4.5.3.1.6.1. 3'-Azido-2',3'-dideoxy-5'-O-(10-phenyldecanoyl)thymidine (86)



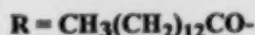
Oil; yield (519 mg, 62%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.42 (br m, 10H, methylene envelope), 1.52-1.74 (m, 4H,  $\text{CH}_2\text{CH}_2\text{Ph}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.36 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.27-2.42 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2'',3'} = 7.5$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.48 (ddd,  $J_{\text{gem}} = 13.9$ ,  $J_{2',3'} = 6.2$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 2.59 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{Ph}$ ), 4.07 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.5$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.19 (ddd,  $J_{3',2''} = 7.5$ ,  $J_{3',2'} = 6.2$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.29 and 4.38 (two dd,  $J_{\text{gem}} = 12.1$ ,  $J_{5'',4'} = 4.5$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.12 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.22 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 7.10-7.20 (m,



2H, aromatic), 7.20-7.36 (m, 3H, aromatic), 9.64 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.54 (5- $\text{CH}_3$ ), 24.73, 29.01, 29.11, 29.16, 29.23, 29.31, 31.35 (methylene carbons), 34.01 ( $\text{CH}_2\text{COO}$ ), 35.85 ( $\text{CH}_2\text{Ph}$ ), 37.52 (C-2'), 60.59 (C-3'), 63.11 (C-5'), 81.73 (C-4'), 85.42 (C-1'), 111.21 (C-5), 125.47, 128.13, 128.29 (aromatic carbons), 135.14 (C-6), 142.77 (aromatic quaternary), 150.17 (C-2 C=O), 163.75 (C-4 C=O), 173.04 (COO).

*Anal.* Calcd. for  $\text{C}_{26}\text{H}_{35}\text{N}_5\text{O}_5$ : C 62.76, H 7.09, N 14.07; found C 63.15, H 7.42, N 13.68.

#### 4.5.3.1.6.2. 3'-Azido-2',3'-dideoxy-5'-*O*-myristoyl thymidine (87)



**Method a:** A mixture of 1,1'-carbonyldiimidazole (206 mg, 0.7 mmol) and methyl iodide (81  $\mu\text{L}$ , 1.3 mmol) was stirred in dry acetonitrile (15 mL) for 2 h at room temperature. Myristic acid (148 mg, 0.7 mmol) was added to the solution in 15 min (carbon dioxide gas was generated immediately) followed by AZT (174 mg, 0.7 mmol). The mixture was stirred at room temperature for 39 hr. The reaction solution was concentrated under reduced pressure. The residue consisting of one major product was purified by chromatography over silica gel using first hexane, and then chloroform, as eluent to yield 198 mg (64%) of product (87) as a colorless oil.

**Method b:** Thymidine (8, 0.5 g, 2.1 mmol) and triphenylphosphine (0.8 g, 3.1 mmol) were dissolved in dry DMF (4.1 mL). To this stirred mixture, first a solution of diethyl azodicarboxylate (DEAD) (488  $\mu\text{L}$ , 3.1 mmol) in DMF (0.96 mL), and then a solution of myristic acid (70, 0.7 g, 3.1 mmol) in DMF (0.96 mL) were added drop by drop, and stirring was continued at room temperature for 15 min. The same quantity of  $\text{Ph}_3\text{P}$  and DEAD were then added. After 30 min stirring at room temperature the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel. Elution with 97:3 chloroform-methanol gives 280 mg (31%) of 2,3'-anhydro-1-(5'-*O*-myristoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (88); mp 140-142°C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.81 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.14-1.40 (br m, 20H, methylene envelope), 1.50-1.64 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.93 (d,  $J_{5-\text{CH}_3,6} = 1.0$  Hz, 3H, 5- $\text{CH}_3$ ), 2.28 (t,  $J = 7.5$  Hz, 2H,

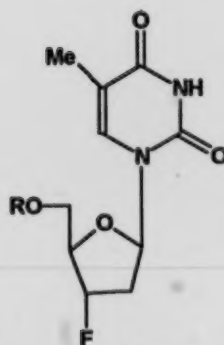
$\text{CH}_2\text{CO}$ ), 2.42-2.52 (ddd,  $J_{\text{gem}} = 12.5$ ,  $J_{2'',1'} = 4.0$ ,  $J_{2'',3'} = 2.9$  Hz, 1H, H-2''), 2.80 (dd,  $J_{\text{gem}} = 12.5$ ,  $J_{2',1'} = 4.0$  Hz, 1H, H-2'), 4.16-4.36 (m, 2H, H-5' and H-5''), 4.36-4.46 (m, 1H, H-4'), 5.20 (br s, 1H, H-3'), 5.53 (d,  $J_{1',2''} = J_{1',2'} = 4.0$  Hz, 1H, H-1'), 6.96 (d,  $J_{6,5-\text{CH}_3} = 1.0$  Hz, 1H, H-6);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  13.39 (5- $\text{CH}_3$ ), 14.07 ( $\text{CH}_3$ ), 22.66, 24.78, 29.08, 29.22, 29.33, 29.43, 29.63, 31.91, 32.92 (methylene carbons), 33.91 ( $\text{CH}_2\text{COO}$ ), 61.75 (C-5'), 82.85 (C-4'), 87.57 (C-1'), 118.80 (C-5), 134.95 (C-6), 153.26 (C-4 C=O), 171.79 (C-2), 173.17 (COO).

*Anal.* calcd. for  $\text{C}_{24}\text{H}_{38}\text{N}_2\text{O}_5$ : C 66.33, H 8.81, N 6.41; found C 65.93, H 8.46, N 6.30.

2,3'-Anhydro-1-(5'-*O*-myristoyl-2'-deoxy- $\beta$ -D-lyxosyl)thymine (88, 198 mg, 0.45 mmol) and sodium azide (126 mg, 1.9 mmol) are suspended in DMF (8.9 mL) and the mixture was heated in the oil bath at 125 °C for 17 h. The orange homogenous mixture was poured onto  $\text{H}_2\text{O}$  (36 mL) and the aqueous layer was extracted with ethyl acetate (2  $\times$  10 mL). After drying ( $\text{Na}_2\text{SO}_4$ ), the solvent was removed *in vacuo* to give a foam-like solid. The residue was purified by silica gel column using chloroform as eluent to yield the product (87, 112 mg, 52%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.86 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.14-1.50 (br m, 20H, methylene envelope), 1.57-1.72 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.93 (d,  $J_{5-\text{CH}_3,6} = 1.1$  Hz, 3H, 5- $\text{CH}_3$ ), 2.36 (t,  $J = 7.4$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.28-2.42 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2'',3'} = 7.1$ ,  $J_{2'',1'} = 6.3$  Hz, 1H, H-2''), 2.47 (ddd,  $J_{\text{gem}} = 13.8$ ,  $J_{2',3'} = 6.5$ ,  $J_{2',1'} = 6.3$  Hz, 1H, H-2'), 4.07 (ddd,  $J_{4',3'} = 4.5$ ,  $J_{4',5''} = 4.2$ ,  $J_{4',5'} = 3.5$  Hz, 1H, H-4'), 4.18 (ddd,  $J_{3',2''} = 7.1$ ,  $J_{3',2'} = 6.5$ ,  $J_{3',4'} = 4.5$  Hz, 1H, H-3'), 4.29 and 4.37 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5'',4'} = 4.2$ ,  $J_{5',4'} = 3.5$  Hz, 1H each, H-5' and H-5''), 6.15 (t,  $J_{1',2''} = J_{1',2'} = 6.3$  Hz, 1H, H-1'), 7.21 (d,  $J_{6,5-\text{CH}_3} = 1.1$  Hz, 1H, H-6), 9.32 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.54 (5- $\text{CH}_3$ ), 14.03 ( $\text{CH}_3$ ), 22.61, 24.82, 29.10, 29.17, 29.28, 29.38, 29.58, 30.37, 31.86 (methylene carbons), 34.08 ( $\text{CH}_2\text{COO}$ ), 37.61 (C-2'), 60.68 (C-3'), 63.15 (C-5'), 81.83 (C-4'), 85.50 (C-1'), 111.27 (C-5), 135.11 (C-6), 150.11 (C-2 C=O), 163.59 (C-4 C=O), 173.07 (COO). *Anal.* Calcd. for  $\text{C}_{24}\text{H}_{39}\text{N}_5\text{O}_5$ : C 60.35, H 8.23, N 14.66; found C 60.21, H 8.47,

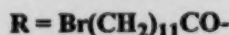
N 14.18.



General Structure

## 4.5.4. 5'-O-ester of FLT

## 4.5.4.1. 3'-Fluoro-2',3'-dideoxy-5'-O-(12-bromododecanoyl)thymidine (91a)

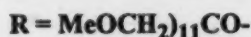


Oil; yield (343 mg, 52%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.50 (br m, 14 H, methylene envelope), 1.58-1.70 (m,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.86 (quintet,  $J = 6.8$ ,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.0$  Hz, 3H, 5- $\text{CH}_3$ ), 2.10 (dddd,  $J_{2'',\text{F}} = 35.5$ ,  $J_{\text{gem}} = 14.5$ ,  $J_{2'',1'} = 8.9$ ,  $J_{2'',3'} = 5.5$  Hz, 1H, H-2''), 2.35 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.69 (ddd,  $J_{2',\text{F}} = 20.5$ ,  $J_{\text{gem}} = 14.5$ ,  $J_{2',1'} = 5.7$  Hz, 1H, H-2'), 3.42 (t,  $J = 6.8$  Hz, 3H,  $\text{CH}_2\text{Br}$ ), 4.27 and 4.40 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5',4'} = 4.3$  Hz, 1H each, H-5'), 4.47 (dt,  $J_{4',\text{F}} = 25.7$ ,  $J_{4',5'} = 4.3$  Hz, 1H, H-4'), 5.18 (dd,  $J_{3',\text{F}} = 52.5$ ,  $J_{3',2''} = 5.5$  Hz, 1H, H-3'), 6.34 (dd,  $J_{1',2''} = 8.9$ ,  $J_{1',2'} = 5.7$  Hz, 1H, H-1'), 7.26 (d,  $J_{6,5-\text{CH}_3} = 1.0$  Hz, 1H, H-6), 9.14 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.60 (5- $\text{CH}_3$ ), 24.77, 26.80, 28.09, 28.66, 28.78, 29.05, 29.13, 29.32 (methylene carbons), 34.08 ( $\text{CH}_2\text{COO}$ ), 38.29 (C-2',  $J_{2',\text{F}} = 21.7$  Hz), 45.10 ( $\text{CH}_2\text{Br}$ ), 63.23 (C-5',  $J_{5',\text{F}} = 10.5$  Hz), 82.43 (C-4',  $J_{4',\text{F}} = 25.72$  Hz), 85.37 (C-1'), 93.40 (C-3',  $J_{3',\text{F}} = 179.3$  Hz), 111.42 (C-5), 134.63 (C-6), 150.12 (C-2 C=O), 163.47 (C-4 C=O), 172.92 (COO).

Anal. Calcd. for  $\text{C}_{22}\text{H}_{34}\text{BrFN}_2\text{O}_5$  C 52.28, H 6.78, N 5.54; found C 52.49, H 7.02, N

5.43.

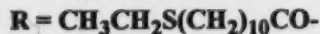
## 4.5.4.2. 3'-Fluoro-2',3'-dideoxy-5'-O-(12-methoxydodecanoyl)thymidine (91b)



Yield (598 mg, 58%), mp = 56-57 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.20-1.42 (br m, 14H, methylene envelope), 1.50-1.72 (m, 4H,  $\text{CH}_2\text{CH}_2\text{O}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.0$  Hz, 3H, 5- $\text{CH}_3$ ), 2.09 (dddd,  $J_{2'',\text{F}} = 36.5$ ,  $J_{\text{gem}} = 14.7$ ,  $J_{2'',1'} = 8.9$ ,  $J_{2'',3'} = 5.4$  Hz, 1H, H-2''), 2.35 (t,  $J = 7.6$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.69 (ddd,  $J_{2',\text{F}} = 20.8$ ,  $J_{\text{gem}} = 14.7$ ,  $J_{2',1'} = 5.4$  Hz, 1H, H-2'), 3.34 (s, 3H,  $\text{OCH}_3$ ), 3.37 (t,  $J = 6.6$  Hz, 2H,  $\text{OCH}_2\text{CH}_2$ ), 4.37 and 4.41 (two dd,  $J_{\text{gem}} = 12.3$ ,  $J_{5',4'} = 4.1$  Hz, 1H each, H-5'), 4.45 (dt,  $J_{4',\text{F}} = 25.5$ ,  $J_{4',5'} = 4.1$  Hz, 1H, H-4'), 5.18 (dd,  $J_{3',\text{F}} = 53.4$ ,  $J_{3',2''} = 5.3$  Hz, 1H, H-3'), 6.34 (dd,  $J_{1',2''} = 8.9$ ,  $J_{1',2'} = 5.4$  Hz, 1H, H-1'), 7.26 (d,  $J_{6,5-\text{CH}_3} = 1.0$  Hz, 1H, H-6), 9.60 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.57 (5- $\text{CH}_3$ ), 24.78, 26.06, 29.05, 29.13, 29.32, 29.40, 29.46, 29.55 (methylene carbons), 34.08 ( $\text{CH}_2\text{COO}$ ), 38.29 (C-2',  $J_{2',\text{F}} = 21.2$  Hz), 58.43 ( $\text{OCH}_3$ ), 63.23 (C-5',  $J_{5',\text{F}} = 9.8$  Hz), 72.99 ( $\text{OCH}_2$ ), 82.43 (C-4',  $J_{4',\text{F}} = 26.7$  Hz), 85.35 (C-1'), 93.41 (C-3',  $J_{3',\text{F}} = 180.3$  Hz), 111.42 (C-5), 134.60 (C-6), 150.18 (C-2 C=O), 163.52 (C-4 C=O), 172.94 (COO).

Anal. Calcd. for  $\text{C}_{23}\text{H}_{37}\text{FN}_2\text{O}_6$  C 60.51, H 8.17, N 6.14; found C 60.28, H 8.43, N 6.12.

## 4.5.4.3. 3'-Fluoro-2',3'-dideoxy-5'-O-(12-thioethyldodecanoyl)thymidine (91c)



Yield (339, 53%), mp = 82-83 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.23-1.48 (br m, 14H, methylene envelope), 1.26 (t,  $J = 7.1$  Hz, 3H,  $\text{CH}_3$ ), 1.50-1.72 (m, 4H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.94 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.09 (dddd,  $J_{2'',\text{F}} = 35.6$ ,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',1'} = 9.0$ ,  $J_{2'',3'} = 5.5$  Hz, 1H, H-2''), 2.34 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.51 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{S}$ ), 2.53 (q,  $J = 7.1$  Hz, 2H,  $\text{CH}_3\text{CH}_2\text{S}$ ), 2.68 (ddd,  $J_{2',\text{F}} = 21.0$ ,  $J_{\text{gem}} = 14.0$ ,  $J_{2',1'} = 5.6$  Hz, 1H, H-2'), 4.26 and 4.39 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5',4'} = 4.0$

Hz, 1H each, H-5'), 4.45 (dt,  $J_{4',F} = 25.5$ ,  $J_{4',5'} = 4.0$  Hz, 1H, H-4'), 5.17 (dd,  $J_{3',F} = 52.5$ ,  $J_{3',2''} = 5.5$  Hz, 1H, H-3'), 6.32 (dd,  $J_{1',2''} = 9.0$ ,  $J_{1',2'} = 5.6$  Hz, 1H, H-1'), 7.24 (d,  $J_{6,5-CH_3} = 1.2$  Hz, 1H, H-6), 8.97 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.63 (5- $CH_3$ ), 14.79 ( $CH_3CH_2S$ ), 24.82, 25.93, 28.90, 29.09, 29.19, 29.37, 29.45, 29.62, 31.68 (methylene carbons), 34.11 ( $CH_2COO$ ), 38.33 (C-2',  $J_{2',F} = 22.1$  Hz), 63.26 (C-5',  $J_{5',F} = 10.1$  Hz), 82.46 (C-4',  $J_{4',F} = 25.7$  Hz), 85.39 (C-1'), 93.40 (C-3',  $J_{3',F} = 179.3$  Hz), 111.45 (C-5), 134.65 (C-6), 150.10 (C-2 C=O), 163.42 (C-4 C=O), 172.97 (COO).  
*Anal.* Calcd. for  $C_{24}H_{39}FN_2O_5S$ : C 59.23, H 8.08, N 5.76; found C 59.52, H 8.37, N 5.62.

#### 4.5.4.4. 3'-Fluoro-2',3'-dideoxy-5'-O-(12-azidododecanoyl)thymidine (91d)



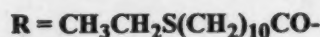
General procedure; oil; yield (556 mg, 91%); Using another method 3'-fluoro-2',3'-dideoxy-5'-O-(12-bromomyristoyl)thymidine (91a, 164 mg, 0.3 mmol) and sodium azide (28 mg, 0.4 mmol) in DMF (3.3 mL) were stirred for 6 h under nitrogen. The solvent was removed *in vacuo* and the residue was dissolved in water (5 mL) and extracted with chloroform (3  $\times$  5 mL). After drying with sodium sulfate and evaporation of chloroform, the residue consisting of one major product was purified by silica gel chromatography using methylene chloride/ethyl acetate 80:20 as eluent to yield 120.5 mg (80%) of product as a colorless foam.  $^1H$  NMR ( $CDCl_3$ )  $\delta$  1.16-1.46 (br m, 14H, methylene envelope), 1.50-1.70 (m, 4H,  $CH_2CH_2CO$ ,  $CH_2CH_2N_3$ ) 1.94 (d,  $J_{5-CH_3,6} = 1.2$  Hz, 3H, 5- $CH_3$ ), 2.10 (dddd,  $J_{2'',F} = 35.6$ ,  $J_{gem} = 14.2$ ,  $J_{2'',1'} = 8.9$ ,  $J_{2'',3'} = 5.2$  Hz, 1H, H-2''), 2.35 (t,  $J = 7.4$  Hz, 2H,  $CH_2CO$ ), 2.69 (ddd,  $J_{2',F} = 21.1$ ,  $J_{gem} = 14.2$ ,  $J_{2',1'} = 5.5$  Hz, 1H, H-2'), 3.26 (t,  $J = 7.8$  Hz, 3H,  $CH_2N_3$ ), 4.27 and 4.40 (two dd,  $J_{gem} = 12.0$ ,  $J_{5',4'} = 3.9$  Hz, 1H each, H-5'), 4.47 (dt,  $J_{4',F} = 26.0$ ,  $J_{4',5'} = 3.9$  Hz, 1H, H-4'), 5.18 (dd,  $J_{3',F} = 53.5$ ,  $J_{3',2''} = 5.2$  Hz, 1H, H-3'), 6.34 (dd,  $J_{1',2''} = 8.9$ ,  $J_{1',2'} = 5.5$  Hz, 1H, H-1'), 7.26 (d,  $J_{6,5-CH_3} = 1.2$  Hz, 1H, H-6), 9.00 (s, 1H, N-H);  $^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.54 (5- $CH_3$ ), 24.73,



26.59, 27.23, 28.72, 29.00, 29.07, 29.26, 29.31 (methylene carbons), 34.02 ( $\text{CH}_2\text{COO}$ ), 38.24 (C-2',  $J_{2',\text{F}} = 21.7$  Hz), 51.39 ( $\text{CH}_2\text{N}_3$ ), 63.21 (C-5',  $J_{5',\text{F}} = 21.7$  Hz), 82.39 (C-4',  $J_{4',\text{F}} = 26.92$  Hz), 85.33 (C-1'), 93.40 (C-3',  $J_{3',\text{F}} = 179.3$  Hz), 111.38 (C-5), 134.63 (C-6), 150.29 (C-2 C=O), 163.72 (C-4 C=O), 172.91 (COO).

*Anal.* Calcd. for  $\text{C}_{24}\text{H}_{34}\text{FN}_5\text{O}_5$ : C 56.52, H 7.32, N 14.98; found C 56.21, H 7.61, N 14.58.

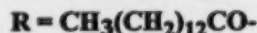
#### 4.5.4.5. 3'-Fluoro-2',3'-dideoxy-5'-O-(11-thioethylundecanoyl)thymidine (92)



Oil; yield (455 mg, 74 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.22-1.44 (br m, 12H, methylene envelope), 1.24 (t,  $J = 7.2$  Hz, 3H,  $\text{CH}_3$ ), 1.50-1.74 (m, 4H,  $\text{CH}_2\text{CH}_2\text{S}$ ,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.93 (d,  $J_{5-\text{CH}_3,6} = 1.3$  Hz, 3H, 5- $\text{CH}_3$ ), 2.08 (dddd,  $J_{2'',\text{F}} = 36.0$ ,  $J_{\text{gem}} = 14.0$ ,  $J_{2'',1'} = 9.0$ ,  $J_{2'',3'} = 4.9$  Hz, 1H, H-2''), 2.34 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.50 (t,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{S}$ ), 2.51 (q,  $J = 7.2$  Hz, 2H,  $\text{CH}_3\text{CH}_2\text{S}$ ), 2.68 (ddd,  $J_{2',\text{F}} = 20.5$ ,  $J_{\text{gem}} = 14.0$ ,  $J_{2',1'} = 5.5$  Hz, 1H, H-2'), 4.25 and 4.38 (two dd,  $J_{\text{gem}} = 12.0$ ,  $J_{5',4'} = 4.1$  Hz, 1H each, H-5'), 4.45 (dt,  $J_{4',\text{F}} = 25.5$ ,  $J_{4',5'} = 4.1$  Hz, 1H, H-4'), 5.16 (dd,  $J_{3',\text{F}} = 52.5$ ,  $J_{3',2''} = 4.9$  Hz, 1H, H-3'), 6.31 (dd,  $J_{1',2''} = 9.0$ ,  $J_{1',2'} = 5.5$  Hz, 1H, H-1'), 7.24 (d,  $J_{6,5-\text{CH}_3} = 1.3$  Hz, 1H, H-6), 8.71 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.56 (5- $\text{CH}_3$ ), 14.73 ( $\text{CH}_3\text{CH}_2\text{S}$ ), 24.73, 25.84, 28.80, 29.00, 29.10, 29.23, 29.31, 29.53, 31.59 (methylene carbons), 34.02 ( $\text{CH}_2\text{COO}$ ), 38.24 (C-2',  $J_{2',\text{F}} = 22.0$  Hz), 63.21 (C-5',  $J_{5',\text{F}} = 10.8$  Hz), 82.39 (C-4',  $J_{4',\text{F}} = 26.9$  Hz), 85.33 (C-1'), 93.40 (C-3',  $J_{3',\text{F}} = 180.4$  Hz), 111.39 (C-5), 134.62 (C-6), 150.25 (C-2 C=O), 163.67 (C-4 C=O), 172.91 (COO).

*Anal.* Calcd. for  $\text{C}_{23}\text{H}_{37}\text{FN}_2\text{O}_5\text{S}$ : C 58.45, H 7.89, N 5.93; found C 58.58, H 8.14, N 5.90.

#### 4.5.4.6. 3'-Fluoro-2',3'-dideoxy-5'-O-(tetradecanoyl)thymidine (93)



Yield (561 mg, 95%); mp = 62.5–64.0 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (t,  $J = 6.6$  Hz, 3H,  $\text{CH}_3$ ), 1.20–1.52 (br m, 20H, methylene envelope), 1.63 (quintet,  $J = 7.5$ ,  $J = 7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 1.93 (d,  $J_{5-\text{CH}_3,6} = 1.0$  Hz, 3H, 5- $\text{CH}_3$ ), 2.10 (dddd,  $J_{2'',\text{F}} = 36.6$ ,  $J_{\text{gem}} = 14.5$ ,  $J_{2'',1'} = 8.9$ ,  $J_{2'',3'} = 4.6$  Hz, 1H, H-2''), 2.34 (t,  $J = 7.5$  Hz, 2H,  $\text{CH}_2\text{CO}$ ), 2.69 (ddd,  $J_{2',\text{F}} = 21.4$ ,  $J_{\text{gem}} = 14.5$ ,  $J_{2',1'} = 5.5$  Hz, 1H, H-2'), 4.27 and 4.38 (two dd,  $J_{\text{gem}} = 12.2$ ,  $J_{5',4'} = 4.0$  Hz, 1H each, H-5'), 4.47 (dt,  $J_{4',\text{F}} = 27.5$ ,  $J_{4',5'} = 4.0$  Hz, 1H, H-4'), 5.18 (dd,  $J_{3',\text{F}} = 53.1$ ,  $J_{3',2''} = 4.6$  Hz, 1H, H-3'), 6.33 (dd,  $J_{1',2''} = 8.9$ ,  $J_{1',2'} = 5.5$  Hz, 1H, H-1'), 7.27 (d,  $J_{6,5-\text{CH}_3} = 1.0$  Hz, 1H, H-6), 9.45 (s, 1H, N-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.59 (5- $\text{CH}_3$ ), 14.09 ( $\text{CH}_3$ ), 22.71, 24.81, 24.91, 29.17, 29.25, 29.27, 29.36, 29.46, 29.65, 31.94, 33.95 (methylene carbons), 34.18 ( $\text{CH}_2\text{COO}$ ), 38.41 (C-2',  $J_{2',\text{F}} = 22.0$  Hz), 63.23 (C-5',  $J_{5',\text{F}} = 9.9$  Hz), 82.56 (C-4',  $J_{4',\text{F}} = 26.4$  Hz), 85.55 (C-1'), 93.39 (C-3',  $J_{3',\text{F}} = 180.18$  Hz), 111.39 (C-5), 134.74 (C-6), 150.10 (C-2 C=O), 163.63 (C-4 C=O), 172.87 (COO).  $^{19}\text{F}$  NMR ( $\text{CDCl}_3$ ) ( $\text{C}_6\text{F}_6$  external std)  $\delta$  -13.04 (dddd,  $J_{\text{F},3'} = 53.1$ ,  $J_{\text{F},2''} = 36.6$ ,  $J_{\text{F},4'} = 27.5$ ,  $J_{\text{F},2'} = 21.4$  Hz, 1F, F-3').

Anal. Calcd. for  $\text{C}_{24}\text{H}_{39}\text{FN}_2\text{O}_5$ : C 63.41, H 8.65, N 6.16; found C 63.71, H 8.70, N 6.16.

### 4.6. Pharmacological assays

#### 4.6.1. *In vitro* antifungal assay

The antifungal assay was carried out in collaboration with Synphar the laboratory of Edmonton.

*Aspergillus niger* PLM 1140, *Candida albicans* ATCC 14053, *Cryptococcus neoformans* KF-33 and *Saccharomyces cerevisiae* PLM 454 were used in the anti-fungal screens in RPMI 1640 growth medium. The broth dilution method adopted from Barchiesi *et al* (1994), was used for determination of minimum inhibitory concentrations (MIC values). Compounds were tested against *A. niger*, *C. albicans*, *C. neoformans* and *S.*

*cerevisiae* in RPMI 1640 growth medium. Various controls, including a standard drug control, solvent control, organism control and medium control were used to ensure the accuracy of the method. The working suspension of the inoculum, prepared spectrophotometrically (the cell density was adjusted by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at a 530 nm wavelength) was made by a 1:50 dilution followed by a 1:20 dilution in RPMI 1640 media to obtain the final suspension. In this method the first well contains 2.5 mg/mL of the compound (25  $\mu$ L of the compound solution in 75  $\mu$ L of the broth), and the next seven microwells have the two-fold diluted solution of the first tube. Then, to each microwell, 100  $\mu$ L of the inoculum was added to reach a total volume of 200  $\mu$ L per well. One hundred microliters of the yeast or fungi suspension was added to each well, resulting in the desired final drug concentration and inoculum size. The microplates were incubated at 35 °C and read at 24 and 48 h for all the isolates except *C. neoformans*, which was read at 48 and 72 h. The microdilution wells were scored with the aid of a reading mirror. The growth in each well was compared with that of the growth control (drug-free) well.

#### 4.6.2. *In vitro* anti-HIV assay

The ability of the test compounds (fatty acids and prodrug esters of dThd, AZT and FLT) to protect HIV-1 infected CD4 lymphocytes (CEM cells) was determined by the United States National Institutes of Health (NIH) testing service using the reported procedure (Weislow *et al.*, 1989). A number of the fatty acids (66b-d, 66i-j, 67d-f) described have not been tested previously against HIV. Some of the fatty acids (65c-d, 66e, 66h, 67a, 68c, 70), for which *in vitro* anti-HIV test results have been reported (Bryant *et al.*, 1989, 1991), were evaluated using assay procedures that are different from those used in this study. The syncytium assay described by Nara *et al* (1987) was used in the NIH anti-HIV screen for quantitation of the number of virus-producing cells in an infected H9 culture where 80-90% of these cells were producing virus. Typical concentrations of infectious virus were  $3 \times 10^5$  syncytium-forming units (SFU)/mL for the III<sub>b</sub> variant of HIV-1 and  $5 \times 10^5$  SFU/mL for the RF variant.

Sensitivity to the lytic effects of HIV-1 infection was determined in preliminary experiments for each host cell line by titrations of cell-free virus or H9 cells chronically infected with HIV-1. In studies employing cell-free virus infections, the amount of virus added was such that the final multiplicity of infection (MOI; ratio of number of infectious virus particles/number of target cells) was the lowest MOI yielding 70% suppression of XTT formazan production in host cells at 7 days. In the cell-free virus assays the MOIs, which were established by syncytium analysis, were 0.1 for CEM-SS.

1. All stock solutions were prepared in 100% dimethyl sulfoxide at the highest achievable concentration for each agent, then diluted 1:100 in cell culture medium before preparing serial half-log<sub>10</sub> dilutions. CD4 lymphocytes (CEM cell line) were added, and after a brief interval HIV-1 was added, resulting in a 1:200 final dilution of the compound. Initial drug dilutions (1:200) resulted in a culture maximum concentration for dimethyl sulfoxide which had no apparent direct toxic effects on the cell lines used or the HIV-1 infection.

2. Cultures were incubated at 37 °C in a 5% carbon dioxide atmosphere for 6 days.

3. The tetazolium salt, XTT, was added to all wells, and cultures were incubated to allow formazan color development by viable cells.

4. Individual wells were analyzed spectrophotometrically ( $\lambda = 450$  nm) to quantitate formazan production, and also viewed microscopically for detection of viable cells and confirmation of protective activity.

5. Drug-treated virus-infected cells were compared with drug-treated noninfected cells and with other appropriate controls (untreated infected and untreated noninfected cells, drug-containing wells without cells, etc.) on the same plate.

6. Data were reviewed in comparison with other tests done at the same time and a determination of activity was made.

In this assay, agents that interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. Nine different concentrations in the range of 0.064 -200  $\mu$ M were used for each compound. The data for all compounds were compared with a positive (AZT-treated) control performed at the same time under identical conditions. The EC<sub>50</sub> is defined as the 50% antiviral effective concentration,



required to produce a 50% reduction in the cytopathic HIV effect in CD4 lymphocytes. The  $IC_{50}$  value is the drug concentration which results in a 50% survival of uninfected untreated control CEM cells.

#### **4.6.3. *In vitro* Anti-HBV Assays**

Two assay methods were used to determine activity against HBV.

##### **4.6.3.1. Analysis of test compounds against HBV replication in cultures of 2.2.15 cells**

A screening service was provided by the United States NIH (Korba *et al.*, 1991). The protocol for assaying anti-HBV activity in cultures of 2.2.15 cells is briefly summarized as follows. Chronically HBV-producing human liver cells (Acs, *et al.*, 1987) were seeded into 24 well tissue culture plates and grown to confluence. Test compounds were then added daily for a continuous 9 day period. Culture medium (changed daily during the treatment period) was collected and stored for analysis of extracellular (virion) HBV DNA after 0, 3, 6, and 9 days of treatment. Treated cells were lysed for 24 hours following day 9 of treatment for the analysis of intracellular HBV genomic forms. HBV DNA was then analyzed in a quantitative and qualitative manner for overall levels of HBV DNA (both extracellular and intracellular DNA) and the relative rate of HBV replication (intracellular DNA).

##### **4.6.3.1.1. Assay parameters**

Both intracellular and extracellular HBV DNA were analyzed in order to (i) allow for verification of compound efficacy, and (ii) provide potential data on the target site in the HBV replication pathway for the compound based on examination of viral replicative forms. The culture medium was changed daily during the treatment period to (i) prevent the buildup of potentially toxic metabolites derived from the test compound, and (ii) provide an analysis of HBV virion production during discrete 24-hour intervals which enables a quantitative comparison of any effect on virion production. The analysis of HBV DNA was performed using blot hybridization techniques (southern and slot blot) and



[<sup>32</sup>P]-labelled HBV-specific probes. HBV DNA levels were measured by comparison to known amounts of HBV DNA standards applied to each nitrocellulose membrane (gel or slot blot). An AMBIS beta scanner, which measures the radioactive decay of the hybridized probes directly on the nitrocellulose membranes, was used for the quantitative analysis. Standard curves, generated by multiple analyses, were used to correlate CPM measurements made by the beta scanner with relative levels of target HBV DNA. The levels of HBV virion DNA released into the culture medium were analyzed by a slot blot hybridization procedure. HBV DNA levels were then compared to those at Day 0 to determine the effect of drug treatment.

#### **4.6.3.2. *In vitro* assay for screening drugs against hepatitis B virus (HBV) and duck hepatitis B virus (DHBV)**

This screening service was provided by Dr. D. L. Tyrrell's laboratory (Department of Medical Microbiology and Immunology at the University of Alberta) based on the previously reported methods (Korba *et al.*, 1991). The protocol for assaying anti-HBV activity in cultures of 2.2.15 cells is briefly summarized as follows.

##### **4.6.3.2.1. Ducks**

Congenital fertilized Peking duck eggs were obtained from a duck colony maintained at the University of Alberta farm that were stored in a 37 °C egg incubator until hatching. Sera from newly hatched ducklings were screened for the presence of DHBV DNA by dot hybridization (Lee *et al.*, 1989).

##### **4.6.3.2.2. Cell cultures**

1) Primary duck hepatocytes: Primary cultures of duck hepatocytes were prepared from 9-14 day old DHBV ducklings using a modified method of Tuttleman *et al.*, (1986). Cells were cultured in 60 mm cell culture dishes in 4 mL L-15 medium containing 5% fetal bovine serum, penicillin G sodium 10 IU/mL, streptomycin sulfate 10 µg/mL and nystatin 25 units/mL.

2) HepG2 cell culture: The hepatoblastoma cell line HepG2 was obtained from the

American type cell culture collection (ATCC) (Rockville, MD). The monolayer cultures were grown in MEM supplemented with 750 mg/L sodium bicarbonate, 300 mg/L L-glutamine, 50 IU/mL penicillin G sodium, 10 µg/mL streptomycin sulfate, and 10% fetal bovine serum. These cell cultures had no HBV DNA, treated as negative control.

3) 2.2.15 cell culture: The HBV-producing 2.2.15 cell cultures were obtained from Dr. M. A. Sells. These cultures were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistance sequences and two head-to-tail dimers of the HBV genome (Sells *et al.*, 1987). The cells were found to produce elevated levels of HBeAg and HBsAg, secrete infectious virions into the culture medium and contain chromosomally integrated HBV DNA sequence, as well as relaxed circular, covalently closed incomplete episomal copies of the genome, (Acs *et al.*, 1987, Sells *et al.*, 1987, 1988). The 2.2.15 cells were grown in MEM containing 750 mg/L sodium bicarbonate, 300 mg/L L-glutamine, 380 mg/L geneticin sulfate and 10% fetal bovine serum, that were maintained in a 37 °C humidified incubator under a 5% CO<sub>2</sub> atmosphere.

Both HepG2 and 2.2.15 cells were seeded in 6 well culture dishes at 10<sup>6</sup> cells/dish (3 mL cell suspension) and the medium was changed every other day.

#### 4.6.3.2.3. Drug treatment

The test compound was added to the hepatocyte cultures on day two and maintained in culture with media changed every second day until day twelve. Cells were harvested at day fourteen. The test compound was screened at a 10 µM final concentration.

The test compound was added to 2.2.15 cell cultures when the cell monolayers were about 80% confluent, usually after about four days. The cultures were treated with the test compound every second day at 10 µg/mL for 12 days, and cells were harvested on day 14.

#### 4.6.3.2.4. Isolation of DNA from hepatocyte cultures

The hepatocytes were lysed with 1 mL lysis buffer containing 10 mM Tris.HCL (pH = 7.8), 5 mM EDTA (pH = 8.0), 150 mM NaCl and 1% SDS. The lysate was digested with 0.5 mg/mL Proteinase K at 42 °C overnight and deproteinized by extraction with an equal

volume of phenol saturated with 20 mM Tris.HCl (pH = 7.5), 0.5 mM EDTA and 0.1% 8-hydroxyquinoline followed by a extraction with chloroform. Concentrated NaCl (5 M) was added to the aqueous phase to yield a final 0.1 M NaCl concentration and the DNA was precipitated with 2 volumes of 95% ethanol. The DNA pellet was washed with 70% ethanol and dried. The dried DNA was dissolved in 100  $\mu$ L of a solution containing 10 mM Tris.HCl (pH = 7.5) and 1 mM EDTA.

#### 4.6.3.2.5. Isolation of cytoplasmic HBV core particles from 2.2.15 cells

The procedure reported by Staprans *et al.*, (1991) was used with some modifications. Cell monolayers were washed with PBS and incubated at room temperature for 20 minutes with 1 mL lysis buffer (10 mM Tris.HCl pH = 7.5, 1 mM EDTA pH = 8.0, 50 mM NaCl-8% Sucrose-0.25% Nonidet P-40) and then centrifuged for 3 minutes using an Eppendorf microcentrifuge at 4 °C to remove debris and cell nuclei. To the supernatant was added  $MgCl_2$  to give a final 6 mM concentration that was treated for 45 minutes at 37 °C with 20  $\mu$ g/mL DNase 1 and 20  $\mu$ g/mL RNase A. The viral particles contained in each 1 mL sample were precipitated by addition of 0.33 mL of 26% polyethylene glycol in 1.4 M NaCl, 0.025 M EDTA. After 30 minutes on ice, the pellets were resuspended in 0.2 mL of 10 mM Tris.HCl, 5 mM EDTA and 1% SDS (pH = 7.5) and the samples were incubated at 42 °C overnight with 500  $\mu$ g/mL final Proteinase K. The deproteinized and ethanol precipitation steps were the same as previous described.

#### 4.6.3.2.6. Dot hybridization

DNA samples were applied to a nylon filter (Hybond-N, Amersham) using a Bio-Dot<sup>TM</sup> (Bio-Rad laboratories) microfiltration apparatus. DNA on the filter was denatured with 0.5 M NaOH, 1.5 M NaCl at room temperature for 30 minutes and neutralized in 1 M Tris.HCl (pH = 8.0), 1.5 M NaCl. The filters were exposed to ultraviolet irradiation for 3 minutes (UV transilluminator, UVP, Inc; 254 nm, total exposure = 1.44 J/cm<sup>2</sup>). Prior to hybridization, the filter was prehybridized for 2 hrs at 65 °C in 5  $\times$  SSPE (20 SSPE is 3.6

M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub>, pH = 7.4, 20 mM EDTA), 5 × Denhardt's (50 × Denhardt's solution contains 10 g of Ficoll, 10 g of polyvinylpyrrolidone, 10 g of bovine serum albumin dissolved in water to a final volume of 1 L), 0.1% SDS, 100 µg/mL denatured salmon sperm.

DNA hybridization was initiated by adding recently prepared DHBV <sup>32</sup>P DNA probe at  $1 \times 10^6$  cpm/mL using the same prehybridization procedure overnight. Filters were washed twice in 1 × SSC (20 × SSC is 3 M NaCl, 0.3 M sodium citrate, pH = 7.0), 0.1% SDS at 65 °C for 2 hrs and 1 × SSC at room temperature for 30 minutes with gentle, constant agitation. The filter was dried and autoradiographed at -70 °C using X-ray film (Kodak X-OMAT AR) with an enhancer screen. After an autoradiographic image had been obtained, the filter was exposed in phosphoimaging screen for 1-2 hrs, and samples were quantitated by Fujix BAS1000 (Fuji Inc.) and the percentage density of phosphoimaging units was calculated.

(-)-Beta-L-2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) was used as the reference compound. Tests were repeated twice and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. Hep G2 cells and the culture medium, which do not hybridize to the HBV DNA probe, were used in all subsequent experiments as a negative control. Four dilutions of the test compound were used in the anti-HBV assay at different concentration ranges. A 10 µg/mL concentration of the myristic acid analog was employed for the anti-DHBV assay and each experiment was performed in triplicate.

$$\frac{[\text{Sample (treated 2.2.15 sample)} - \text{Negative Control (HepG2)}] \times 100}{[\text{Untreated 2.2.15 Positive Control} - \text{Negative Control (HepG2)}]} = \% \text{ of replication}$$

or 
$$\frac{[\text{Treated Hepatocyte Sample} - \text{Negative Hepatocyte}] \times 100}{[\text{Congenital Untreated Hepatocyte} - \text{Negative Hepatocyte}]} = \% \text{ of replication.}$$

#### 4.6.4. *In vitro* cytotoxicity assays

Cytotoxicity assays were performed using different cell lines and methods:

a. A CD4<sup>+</sup> lymphocyte CEM cell line was used according to the United States National Cancer Institute procedure (Weislow *et al.*, 1989). This assay determines the percentage of surviving uninfected cells exposed to the test compound relative to uninfected, unexposed controls using the tetrazolium salt (XTT) assay. XTT was added to the cell culture and samples were analyzed spectrophotometrically ( $\lambda = 450$  nm) to quantitate formazan production. The experimental details were previously described in section 4.6.2.

b. The protocol for determining toxicity of test compounds in cultures of 2.2.15 cells can be briefly summarized as follows (Korba and Gerin, 1992). 2.2.15 cells were grown to confluence in 96 well flat-bottomed tissue culture plates and treated with test compound (in 0.2 mL culture medium/well) as described above. Four concentrations of each test compound were assayed, each in triplicate cultures, in 3 to 10-fold dilution steps. Untreated control cultures were maintained on each 96 well plate. On each 96 well plate, wells containing no cells were used to correct for light scattering. Toxicity was determined from the inhibition of neutral red dye uptake, as determined from the absorbance at 510 nm relative to untreated cells, at 24 hours following day 9 of treatment.

c. *In vitro* KB cell cytotoxicity (TD<sub>50</sub>,  $\mu\text{g/mL}$ ) was determined for a few compounds using the tetrazolium salt (MTT) method which involves conversion of MTT to colored formazan according to previously reported methods (Hanson *et al.*, 1989, Parang *et al.*, 1996).

#### 4.7. Analytical Systems

##### 4.7.1. Chromatography

Thin layer chromatography (TLC) was performed using Whatman MK6F silica gel microslides (250  $\mu\text{m}$  thickness). Reversed phase thin layer chromatography was carried out using Whatman LKC18F linear-K reversed phase silica gel microslides (5  $\times$  20 cm, 200  $\mu\text{m}$  thickness).



A measurement of the lipophilicity of the fatty acids of interest was carried out chromatographically on TLC plates (K6F Silica Gel 60 A°, Size: 10 × 20 cm, Layer thickness: 250  $\mu$ , Whatman).  $R_f$  values (mean  $\pm$  SD) was measured using the mobile phase: diethyl ether/hexane (50 mL, 90:10). The plates were developed by bromocresol green or primuline.

Silica gel column chromatography was carried out using Merck 7734 (60-200 mesh) silica gel.

Preparative thin layer chromatography (PTLC) was carried out using Whatman PLK5F plates (1 mm thickness).

#### 4.7.2. High performance liquid chromatography

Quantitative and qualitative analyses of samples for all experiments described were performed using a high performance liquid chromatography (HPLC) system comprised of a Waters baseline 810 computer program operating on a 486/33 MHz computer. A Waters model 501 pump, Waters model U6K injector, Hewlett Packard 1040A photodiode-detector or Waters 486 variable wavelength detector and a HP 79994A workstation or a Waters system interface module were used for HPLC analysis. The UV detector was set to monitor absorbance at 265 nm. An evaporative light scattering detector (ELSD) (Varex MK III, Alltech Association, Inc.) was used for detecting the fatty acids at different temperatures and flow gas rates. Organic solvents (methanol and acetonitrile) and water used as mobile phases were HPLC grade and filtered through a 0.45  $\mu$ M (water) and 0.25  $\mu$ M (organic solvents) filter (Millipore).

##### 4.7.2.1. HPLC conditions

Several HPLC conditions were designed for use with various compounds and various purposes.

##### 4.7.2.1.1. *In vitro* studies and retention times of esters

HPLC analyses for *in vitro* studies of the esters and measurement of retention times for the esters were performed using a Waters Radial-Pak C18 reverse phase cartridge

column (8 mm ID  $\times$  10 cm length, 10  $\mu$ m particle size) at 25  $^{\circ}$ C with methanol:water (75:25, v/v) for retention time measurements and methanol:water or acetonitrile:water (70:30, v/v) for *in vitro* studies, at a flow rate of 1 mL/min.

#### 4.7.2.1.2. Retention times of fatty acids

Retention times (mean  $\pm$  SD) for fatty acids were measured by HPLC using a Waters Radial-Pak C18 reverse phase cartridge column (8 mm ID  $\times$  10 cm length, 10  $\mu$ m particle size) and evaporative light scattering detector (ELSD) using conditions: acetonitrile:water: 90/10, flow rate = 1 mL/min, drift tube temperature = 102  $\pm$  0.2  $^{\circ}$ C, gas flow = 2.15 SLPM, solvent press = 7.2-7.4 PSIG.

#### 4.7.2.1.3. *In vivo* study of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) and 3'-azido-2',3'-dideoxythymidine (6)

HPLC analyses for the *in vivo* studies of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) and 3'-azido-2',3'-dideoxythymidine (6) were carried out using a Phenomenex Partisil 5 ODS(3) (250  $\times$  4.60 mm, 5 micron particle size) column using a gradient system. In order to separate internal standard, AZT and the ester prodrug, a complex gradient of acetonitrile and water was employed. Details of the separation are shown in Table 4.1. The prodrug (85b), AZT and internal standard peak were well separated and no interfering peaks were observed. The retention times for 5-bromo-2'-deoxyuridine (internal standard), AZT and ester were 12.3, 22.3 and 49.9 min, respectively. The identity of each compound present in the sample was determined by comparison of its retention time to that of an authentic sample. The gradient system used is as follows:

| <u>Time (min)</u> | <u>%Acetonitrile</u> | <u>%Water</u> |
|-------------------|----------------------|---------------|
| 0                 | 5                    | 95            |
| 17                | 20                   | 80            |
| 19                | 60                   | 40            |
| 22-35             | 70                   | 30            |
| 40-70             | 90                   | 10            |
| 85                | 5                    | 95            |

Table 4.1. Gradient system used for analyzing 6 and 85b samples for the *in vivo* study.

The lower limit of detection is defined as the smallest concentration that can be reliably distinguished from zero. It was determined to be  $1.5 \times 10^{-9}$   $\mu\text{mol}/\mu\text{L}$  (0.0004  $\mu\text{g}/\text{mL}$ ),  $4.9 \times 10^{-4}$   $\mu\text{mol}/\text{g}$  (0.13  $\mu\text{g}/\text{g}$ ),  $1.8 \times 10^{-3}$   $\mu\text{mol}/\text{g}$  (0.48  $\mu\text{g}/\text{g}$ ), for blood, liver and brain for AZT, respectively. The detection limit for 85b determined to be  $3.3 \times 10^{-9}$   $\mu\text{mol}/\mu\text{L}$  (0.0018  $\mu\text{g}/\text{mL}$ ) and  $4.8 \times 10^{-4}$   $\mu\text{mol}/\text{g}$  (0.27  $\mu\text{g}/\text{g}$ ) for blood and liver, respectively. Quantification of compounds was performed by measuring the AUC of the test compound (6 and 85b) relative to that of the internal standard. Standard curve slopes were generated using a square linear regression. The standard curve was linear. The limit of quantification for each compound was based on a signal to noise ratio of 3:1. The precision of the analytical methods assessed by intra-day and inter-day relative standard deviations were less than 10% at all prodrug concentrations.

#### 4.7.2.1.4. *In vivo* study of 2-bromotetradecanoic acid (68c)

HPLC analysis of samples from *in vivo* studies of 2-bromotetradecanoic acid (68c) produced from the prodrug ester (85b) or 2-bromotetradecanoic acid (68c) administration was carried out using a Waters Radial-Pak C18 reverse phase cartridge column (8 mm ID  $\times$  10 cm length, 10  $\mu\text{m}$  particle size) and a gradient system of acetonitrile/water, flow rate = 1 mL/min, drift tube temperature =  $100 \pm 0.2$   $^{\circ}\text{C}$ , gas flow = 3.12 SLPM, solvent pressure = 7.2-7.4 PSIG. Retention times for AZT, 5-bromo-2'-deoxyuridine and 2-bromomyristic acid (68c) were 3.3, 4.5 and 27 min, respectively using these conditions (Table 4.2).

| <u>Time (min)</u> | <u>%Acetonitrile</u> | <u>%Water</u> |
|-------------------|----------------------|---------------|
| 0-14              | 20                   | 80            |
| 16                | 30                   | 70            |
| 17                | 40                   | 60            |
| 19-35             | 50                   | 50            |
| 37                | 40                   | 60            |
| 38                | 30                   | 70            |
| 40                | 20                   | 80            |

Table 4.2. Gradient system used for analyzing 68c samples for the *in vivo* study.

#### 4.8. Physicochemical calculations

##### 4.8.1. Determination of partition coefficients (Log P)

Partition coefficients were determined using a *n*-octanol/phosphate-buffered solution (0.2 M, pH = 7.4) system, with mechanical shaking (Dubnoff metabolic-shaking incubator, Precision Scientific Co.) to ensure equilibration, followed by ultra-violet (UV) quantitation of the analyte in the *n*-octanol phase. *n*-Octanol was refluxed for 4 h prior to distillation and was collected at 196 °C. *n*-Octanol and buffer were mutually saturated by overnight stirring of equal volumes of octanol and buffer. The two phases were then separated using a separatory funnel and each fraction was stored in a brown bottle at room temperature for subsequent use. Standard solutions of the test compound were prepared in *n*-octanol-saturated water. These standard samples were analyzed by UV spectrophotometry using a wavelength of 265 nm. Standard curves were prepared by plotting UV absorbance vs concentration of the standard solutions.

The test compound (four different concentrations 0.15-0.96 mg/5 mL) was partitioned between presaturated *n*-octanol (5 mL) and phosphate buffer (10 mL, 0.2 M, pH = 7.4) for 21 h at room temperature using a mechanical shaker. The two phases were then separated. The concentration of the test compound in the *n*-octanol phase, before and after phosphate buffer partitioning, was determined using the procedure (UV quantitation at 265 nm) reported previously (Tandon *et al.*, 1992). Partition coefficients (P) were calculated as the ratio of the concentration in the *n*-octanol (moles/volume) to the concentration in the phosphate buffer phase (moles/volume) ( $P = C_{n\text{-octanol}}/C_{\text{water}}$ ).

##### 4.8.2. Calculation of physicochemical properties

Physicochemical properties were estimated using the PALLAS computational program (PrologD 2.0 for windows, *pKalc* 3.1 for windows, PrologP 5.1 for windows 1995, Compudrug Chemistry Ltd, Hollán Ernő utca 5., H-1136 Budapest, Hungary). *pKalc* 3.1, an add-on module of the PALLAS system, estimates acidity/basicity and calculates *pKa* values, even for substances that have low water solubility or are unstable in water. The



validity of calculated data in the case of fatty acids was established by comparing the experimental values of a few fatty acids with their  $pK_a$  values (Parang *et al.*, 1996). The PrologP 5.1 program module was used to calculate the Log P values for myristic acid analogs and myristate esters in an *n*-octanol/water system based on their chemical structure. To confirm the validity of the Log P calculations, experimental data were compared with calculated data for selected fatty acids (Parang *et al.*, 1996). The PrologD 2.0 program module was used to predict the logarithm of the distribution coefficient, Log  $D_{7.4}$  and Log D from the compound structure. The distribution coefficient (D) considers the partitioning of all ionic species of a compound. In order to calculate the Log  $D_{7.4}$  and Log  $D_{7.0}$  value for a test compound, both the logarithms of the partition coefficient (Log P) and the dissociation constant(s) ( $pK_a$ ) are needed. To perform these calculations, the test compound structure is drawn graphically, after which the computational program PrologD automatically calculates  $pK_a$ , Log P and Log  $D_{7.4}$  by activating the  $pK_{alc}$ , PrologP and PrologD modules of PALLAS.

#### 4.9. Molecular mechanics calculations and quantitative structure-activity relationships (QSARs)

Molecular mechanics modeling and calculations were performed using the Hyperchem program (Version 4.0, 1994, Hypercube, Inc., 419 Phillip Street, Waterloo, Ontario, Canada N2L 3X2). A QSAR equation was established utilizing the *SciQSAR* program (Sci Vision, 128 Spring St., Lexington, MA 02173) which uses Hyperchem to compute low energy conformations of molecules. Interatomic distances between the CO and the terminal heteroatom moiety were measured after AM1 calculations using the Hyperchem Release 4.0 computational program.

Published X-ray crystal structure data for fatty acids (Hernqvist, 1988), dThd (Young *et al.*, 1969) and 5'-O-acetyl-dThd (Sato, 1988) were used as starting coordinates for molecular optimization. The structures for 5'-O-acetylthymidine and the fatty acids were optimized using a MM+ molecular mechanics force field (Allinger, 1977), and the structural properties (bond distances, dihedral and torsion angles) of optimized 5'-O-acetylthymidine and the fatty acids were combined to establish the starting conformation



to model the respective 5'-O-acylThd esters (81a-h). These calculations were performed in four steps. The first stage in the calculation was to optimize the preliminary structure of the fatty acids and 5'-O-acetylthymidine using the MM+ molecular mechanics force field based on available literature data (Hernqvist, 1988). X-ray crystal structure data for 5'-O-acetylthymidine was also used as the starting point for the nucleoside moiety in these calculations (Sato, 1988). In the second stage, the structures for the 5'-O-esters of thymidine (81a-h) were modeled based on the optimized crystal structures for 5'-O-acetylthymidine and the fatty acid using the MM+ molecular mechanics Hyperchem program. The bond lengths and bond angles in the optimized structures were used to establish the starting geometry for the molecular mechanics calculations. In the third stage, the combined initial structures (thymidine and fatty acid) were subjected to geometry optimization. The conformations were adjusted to fully minimize the molecular energies taking electrostatic terms into account. These preliminary structures were then optimized using the MM+ molecular mechanics force field and PM3 semi-empirical methods. The criterion for termination of each minimization was an energy change (convergence criteria, RMS) of less than 0.01 kcal/mol for MM+ and 0.2 kcal/mol for PM3 *in vacuo* using the Polak-Ribiere (conjugate gradient) algorithm. Finally, the resulting minimum energy conformations were used in the *SciQSAR* program to obtain the regression equation. *SciQSAR* was used to analyze the antiviral activities for the 5'-O-esters of thymidine (81a-h). This program, which is used to establish a QSAR (Quantitative Structure-Activity Relationship), uses a combination of three dimensional geometry optimized structures obtained from MM+ or a semi-empirical method such as PM3 (Stewart, 1989) and a set of calculations involving molecular descriptors which are in part based on these low energy conformations (the azido group in the compounds 81f-h was optimized in different resonance forms,  $-N-N^+=N$  and  $-N=N^+=N$ ). *SCIQSAR* uses Hyperchem to compute low energy conformations of molecules via MM+ and PM3 which provides information that is extracted to compute molecular descriptors such as Log P, Wiener Index, and Molecular Weight. Irrelevant descriptors are eliminated to create a meaningful regression equation. The regression equation was cross-validated by creating a new regression by randomly removing 10 to 20% of the compounds. The statistical parameters,  $R^2$  and RMSD, of the

new QSAR should be close to those employing the complete data set using the original set of descriptors.

**4.10. *In vitro* incubation studies of 5'-O-myristoyl analogue derivatives of dThd, AZT and FLT (Stability of the ester prodrugs)**

The stability of the ester prodrugs *in vitro* was examined in various media (porcine liver esterase, rat plasma, rat brain homogenate) diluted with an isotonic phosphate buffer, pH 7.4.

**4.10.1. Stability of 5'-O-(12-azidododecanoyl)thymidine (81h) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) upon incubation in cell culture medium (RPMI/10% FBS)**

The stability of 81h and 85b in the cell culture medium, used for determination of anti-HIV-1 activity in the CEM cell line, were examined by incubation of 81h and 85b with RPMI 1640/10% FBS at a concentration of 1 mM at 37 °C for 8 days. At specified time intervals (0.5, 1.0, 2, 4, 8, 20, 43, 67, 93, 117, 140, 163, 189 and 211 h), a 100  $\mu$ L aliquot of the incubation mixture was removed and mixed with an equal volume of ice-cold ethanol. This mixture was centrifuged for 15 min at 12,800 rpm, and a 20  $\mu$ L aliquot was analyzed by HPLC using acetonitrile-water (7:3, v/v) as eluent at a flow rate of 1 mL/min and UV detector at 265 nm.

**4.10.2. Stability of esters in the presence of porcine liver esterase**

Porcine liver esterase (Sigma EC 3.1.1.1) was used to measure the susceptibility of ester prodrugs to enzymatic ester hydrolysis. Stock solutions of all derivatives were prepared in ethanol (98%) to give a concentration of  $4.17 \times 10^{-3}$  M, and 60  $\mu$ L of this stock solution was mixed with 3 units of porcine liver esterase (7.51  $\mu$ L in 1 mL phosphate buffer, pH = 7.4) to give the final concentration of  $2.36 \times 10^{-4}$  M at 37 °C. It was confirmed that 0.06% (v/v) ethanol had no measurable inhibitory effect on the enzyme activity. Aliquots of the solution (100  $\mu$ L), which were withdrawn at 2, 4, 6, 8, 10, 20 and

30 min time intervals, were added to the ice-cold methanol (200  $\mu$ L) in a 1.5 mL microcentrifuge tube to stop the enzymatic reaction and the mixture was vortexed. The resultant mixtures were placed on ice for 10 min before centrifugation at 12,800 rpm for 5 min (1500  $\times$  g). The supernatant layers were filtered through a 0.45  $\mu$ m HV filter (Waters Millipore), and a 150  $\mu$ L aliquot was subjected to high performance liquid chromatography (HPLC) analysis using methanol/water (70/30, v/v), flow rate of 1 mL/min and UV detector at 265 nm. Pseudo first-order rate constants were determined from plots of area under the peak of the ester versus time. Half-lives can be calculated according to equation  $t_{1/2} = 0.693/\text{rate constant}$ .

#### **4.10.3. Preparation of biological medium (rat plasma and brain homogenate) for the kinetic enzyme studies**

Male Sprague Dawley rats weighing 250-280 g were anesthetized with pentobarbital. Blood samples were obtained by heart puncture and collected in heparinized tubes. The blood was centrifuged at 1500  $\times$  g for 15 min, and the resulting plasma was used for the experiments. The resulting plasma was stored at 0-5  $^{\circ}$ C for no more than 30 h before use.

The entire brain was removed after incision of the skull. The brain homogenate was prepared by excising the whole brain. After rinsing with 15 mL saline, and drying on a filter paper, the brain was weighed on a watch glass and homogenized with phosphate buffer (pH = 7.4) (brain:phosphate buffer, 1:5 w/v) using a con-Torque power unit for 10-20 min in a glass homogenizer with a Teflon pestle having radial serrations. The homogenates were centrifuged at 1500  $\times$  g for 10 min, and the supernatant was used for the experiments. The temperature was maintained at 0-5  $^{\circ}$ C during the preparation and storage of the brain homogenate. Again these preparations were kept for no more than 30 h before use.

##### **4.10.3.1. Stability of esters in the presence of tissue preparations (rat plasma and brain homogenate)**

Bioactivation experiments were performed at 37  $^{\circ}$ C in the presence of various enzymic preparations diluted with phosphate buffer. The experiments were initiated by adding the

stock solutions of the ester derivatives in ethanol 98% ( $60\ \mu\text{L}$ ,  $9.04 \times 10^{-3}\ \text{M}$  for plasma,  $0.014\ \text{M}$  for brain homogenate) to the enzymic biological preparations ( $1\ \text{mL}$ , plasma:phosphate buffer 1:9 v/v pH = 7.4, or brain:phosphate buffer, 1:5 w/v) previously equilibrated at  $37\ ^\circ\text{C}$  in a shaker bath, and mixed thoroughly to generate a final concentration of  $5.11 \times 10^{-4}\ \text{M}$  for plasma and  $7.87 \times 10^{-4}\ \text{M}$  for brain homogenate. At appropriate times (2, 4, 6, 8, 10, 12 min for plasma, and 2, 4, 6, 8, 10, 20, 120, 240 min for brain homogenate)  $100\ \mu\text{L}$  of the reaction mixture was withdrawn, the reaction was stopped by adding  $200\ \mu\text{L}$  of ice-cold methanol, and the mixture was vortexed. The resultant mixtures were placed on ice for 10 min before centrifugation at 12,800 rpm ( $1500 \times g$ ) for 5 min. Precipitated proteins were removed by centrifuging, the supernatant layers were filtered through a  $0.45\ \mu\text{m}$  filter, and a  $200\ \mu\text{L}$  aliquot was subjected to high performance liquid chromatography (HPLC) analysis using acetonitrile/water (70/30, v/v), flow rate of  $1\ \text{mL/min}$  with UV detection at 265 nm. The decrease in concentration of the esters due to hydrolysis was followed by high performance liquid chromatography (HPLC) analysis of samples of the reaction mixture taken periodically to determine the pseudo-first-order rate constants. Half-lives can be calculated according to equation  $t_{1/2} = 0.693/\text{rate constant}$ . The change in concentration of AZT and FLT released from the prodrugs upon incubation with brain homogenate and plasma was not possible to follow by HPLC due to interfering peaks.

#### 4.10.4. The stability of 3'-azido-2',3'-dideoxy-5'-O-(myristoyl)thymidine (87) in phosphate buffer

The stability of 3'-azido-2',3'-dideoxy-5'-O-(myristoyl)thymidine (87) was investigated by the presence of same amount of ester in a control experiment where the plasma was replaced with the same volume of buffer. A control experiment was also conducted using mouse plasma that had been boiled for 10 min and then homogenized. The samples were processed as described in section 4.10.3.1.



**4.11. *In vivo* distribution studies and determination of pharmacokinetic parameters for 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and (±)-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) in mice**

The biotransformation and pharmacokinetic parameters for (±)-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) were investigated in mice using 0.31 mmol/kg of the prodrug (85b, 169.8 mg/kg dose), AZT (6, 81.5 mg/kg dose) and 2-bromomyristic acid (68c, 93.7 mg/kg dose) via tail-vein administration. AZT and 2-bromomyristic acid were administered simultaneously in the same solution.

**4.11.1. Selection of internal standard for HPLC**

Several compounds were tested to select the proper internal standard for the *in vivo* experiments. Since chromatographic conditions such as flow rate, temperature, volume of injection etc. are very important when using the area under the peak (AUC) method, it was also necessary to use an internal standard to normalize these variables. Some important facts were considered for the optimum selection. The internal standard should be added before addition of protein precipitating solvents. The unknown concentration must fit the calibration curve and should not be outside the calibration curve. The internal standard method compensates for changes in chromatographic conditions as long as they affect the standard and the sample component equally. The internal standard should have the following characteristics. 1. Should have the same or very close chromatographic properties as the component of the sample. 2. Should never be in the sample either as a component or a metabolite. 3. Should be stable under the chromatographic condition. 4. Should be pure and readily available. 5. Same amount of the internal standard should be used in all experiments. Based on the above criteria, 5-bromo-2'-deoxyuridine was selected as the internal standard (50 mg/10 mL in water).

**4.11.2. Calibration curves**

Blood was drawn into a heparinized syringe from mice by heart puncture after the Balb/c mice (25-30 g) were asphyxiated with carbon dioxide. To 200 µL blood was added



100  $\mu\text{L}$  of AZT (**6**) (in water) at different concentrations between 0.000417-1  $\mu\text{g/mL}$  or 100  $\mu\text{L}$  of 2-bromomyristic acid (in ethanol) between 12.0-61.5  $\mu\text{g/mL}$  or 100  $\mu\text{L}$  of ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-*O*-(2-bromomyristoyl)thymidine (**85b**) (in PEG 200:ethanol: 7:3 v/v) at different concentrations between 0.0018-0.45  $\mu\text{g/mL}$  and 100  $\mu\text{L}$  5-bromo-2'-deoxyuridine (50 mg/10 mL in water) as the internal standard. Acetonitrile:DMSO (800  $\mu\text{L}$ , 92:8, v/v) was added to the mixture after vortexing. In the case of the ester, the internal standard and precipitating solution was added first and then the standard solution of ester was added to prevent any unwanted ester hydrolysis by the tissues. After centrifugation for 30 min at a  $1500 \times g$  (12,800 rpm), the supernatant was collected and stored frozen at  $-22^\circ\text{C}$  in the dark prior to quantitative HPLC analysis. From the supernatant solution, 20  $\mu\text{L}$  was subjected to HPLC analysis.

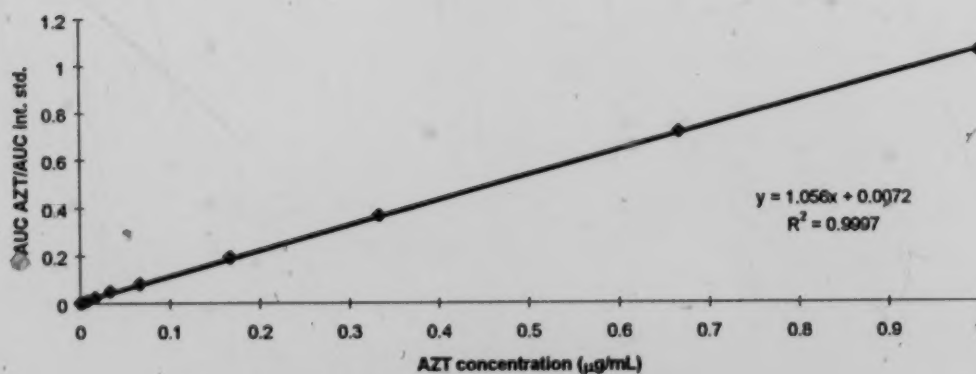


Figure 4.1. Representative calibration curve for AZT in Balb/c mouse blood.

The solid tissues (brain and liver) were removed and quickly frozen on dry ice within 90 s of death. To the weighed tissues was added 300  $\mu\text{L}$  of the AZT solution (in water at different concentrations 0.13-2143.00  $\mu\text{g/g}$  of liver, 0.48-1717.00  $\mu\text{g/g}$  of brain) or 2-bromomyristic acid (in ethanol, 6.1-307.3  $\mu\text{g/g}$  of liver or brain) or ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-*O*-(2-bromomyristoyl)thymidine (**85b**) (in PEG 200:ethanol: 7:3 v/v, 0.27-703.0  $\mu\text{g/g}$ , of liver) and 300  $\mu\text{L}$  internal standard solution (50 mg/10 mL in water). The

mixtures were homogenized in acetonitrile:DMSO (92:8, v/v) (1:2 w/v, brain homogenate:mixed organic solvent) for five min. There was no need for a calibration curve for ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) in brain, since the ester was not detected in brain. In the case of the ester, the internal standard and precipitating solution was added first and then the standard solution of ester was added to prevent any unwanted ester hydrolysis by the tissues. After centrifugation at a  $1500 \times g$  (12,800 rpm) for 15 min, 20  $\mu$ L of supernatant was subjected to HPLC analysis. In all cases for different concentrations, the experiment was carried out in triplicate.

A plot was made of the ratio of peak area for the compound and internal standard, respectively, versus the compound concentration. The sample AUC ratios for AZT/internal standard, fatty acid/internal standard and ester/internal standard were used to calculate concentration from the linear regression equation obtained from standards prepared with mouse blood, liver and brain. Calibration curves were calculated by least square regression analysis. An unknown sample concentration in blood, brain and liver can be determined by using the equations obtained from these calibration curves.

#### 4.11.3. Blood, brain and liver sampling *in vivo* after iv administration of 3'-azido-2',3'-dideoxythymidine (6), 2-bromomyristic acid (68c) and ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b)

The *in vivo* regeneration of AZT from ( $\pm$ )-3'-azido-2',3'-dideoxy-5'-O-(2-bromomyristoyl)thymidine (85b) was studied in mice after iv tail vein injection of the ester. The test compound was dissolved in PEG 200:ethanol (70:30, v/v). The solution was injected into the lateral tail vein of male Balb/c mice weighing 25-30 g over 30 seconds using a dose of 169.8 mg/kg that equivalent to a 81.5 mg/kg dose of AZT. Animals were momentarily restrained during dosing, then placed in individual cages and allowed food and water *ad libitum*. The mice were sacrificed at 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 min post iv injection by asphyxiation with carbon dioxide. For each time point, at least 3 mice were used. Blood was drawn into a heparinized syringe from mice by heart puncture after the Balb/c mice (25-30 g) were asphyxiated with carbon dioxide. Blood samples (700-800  $\mu$ L) were collected in 1.5 mL microcentrifuge Ependorff

tubes. To 200  $\mu\text{L}$  aliquot of blood sample was added 100  $\mu\text{L}$  of 5-bromo-2'-deoxyuridine as internal standard and 100  $\mu\text{L}$  distilled water which was gently agitated. Acetonitrile:DMSO (800  $\mu\text{L}$ , 92:8, v/v) was added to the mixture after vortexing. Centrifugation was performed at  $1500 \times g$  (12,800 rpm) for 30 min, the supernatant was collected and stored frozen at  $-22^\circ\text{C}$  in the dark prior to quantitative HPLC analysis. The analysis of blood contents was carried out using the blood of three mice for each experimental point (each time point being measured in triplicate). From the supernatant, 20  $\mu\text{L}$  was used for such HPLC injection. AZT and the ester were analyzed in blood by a HPLC method using a Phenomenex Partisil 5 ODS 3,  $4.6 \times 250$  mm, RP, C-18 column was used for HPLC analysis using the conditions explained before in section 4.7.2.1.3.

Whole brain and liver samples were removed and placed on dry ice within 90 s of death. After addition of 300  $\mu\text{L}$  distilled water and 300  $\mu\text{L}$  internal standard solution (50 mg/10 mL in water) to the weighed solid tissues (brain and liver), they were weighed and homogenized in acetonitrile:DMSO (92:8, v/v) (1:2 w/v, brain homogenate:mixed organic solvent) for five min.

The above procedure was used for iv administration of AZT (6, 81.5 mg/kg) and 2-bromomyristic acid (68c, 93.7 mg/kg) dissolved together in PEG 200:ethanol (70:30, v/v). The HPLC method for analysis of 2-bromomyristic acid was explained in section 4.7.2.1.4. The same samples were subjected to HPLC chromatography for the analysis of AZT using the method described in section 4.7.2.1.3.

This method allows the collection of many samples without disturbing the blood volume, and the samples are sufficiently clean to allow direct analysis by reverse-phase HPLC without too many purification steps.

#### 4.12. Pharmacokinetics and data analysis

The concentration of AZT (6) and ester (85b) in blood, liver and brain samples as a function of time, was plotted using the Sigmaplot program (Jandel Scientific). The measured mouse blood, liver and brain concentrations were used to calculate the area under the blood or brain concentration-time curve (AUC) from time zero to infinity. Data were analyzed by a two compartmental method using WinNonlin version 1.0

(Scientific Consulting Inc.) for iv bolus input and showed an excellent fit to this model. Blood clearance (CL) was calculated according to the equation  $CL = \text{dose}_{iv} / AUC_{iv}$ .  $AUC_{iv}$  is the value derived from the computer calculation for the area under the blood concentration-time curve. The terminal elimination rate constant was calculated from the slope of the linear regression of the logarithm of blood drug concentrations as function of time in the terminal elimination phase. WinNonlin uses the linear trapezoidal method to calculate area under the concentration (in blood)-time curve (AUC) from time zero to the time of the last sample ( $AUC_{last}$ ) which is extrapolated to infinity. Data are reported as means  $\pm$  standard errors unless stated otherwise. The brain/blood ratio for AZT was compared by use of a multiple-comparison test of mean values.

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